Characterisation of the natural homeotic variety *Stamenoid petals* (*Spe*) in the Shepherd´s Purse (*Capsella bursa-pastoris*) – Establishment of a new model system

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Meinen Eltern und Jorge

Table of contents

1 Introduction

1.1 About homeosis

Studies of homeotic mutants were and are of incredible value for understanding ontogenetic processes in animal and plant model systems in the previous decades. The phenomenon "homeosis" was long debated in history since the first definition of Bateson in 1894 as ´something has been changed into the likeness of something else`. The term underwent numerous refinements, reviewed by Lewis (1994). In segmentally organised organisms one could define homeosis more precisely as segments which show the wrong identity. That is when a segment shows characteristics, which are homologous to characteristics normally displayed in another segment. Defined like that, homeotic transitions constitute an important subset of heterotopic (positional) changes in development (Baum and Donoghue, 2002). A prominent example from one of the earliest investigations of homeotic transitions in animals is the *Ultrabithorax* (*Ubx*) mutant of *Drosophila melanogaster*, where the third segment develops an additional pair of normally developed wings instead of halteres (rudimentary wings). Another long and well known example is the *Antennapedia* (*Antp*) mutant also found in *Drosophila melanogaster*. Here the antennae of the head segment are transformed into an additional pair of legs. Typically these mutants are caused by changes in a gene family encoding for transcription factors which is characterised by a 180 bp sequence element called the homeobox. The corresponding protein domain is responsible for DNA binding. Members of that gene family were therefore called homeobox genes. Both examples are, besides numerous other treatises, reviewed by Gehring and Hiromi (1986), Gehring (1992) and recently by Maeda and Karch (2006).

Homeotic mutants also occur frequently in plants where they effect both vegetative and reproductive organs (Sattler, 1988; Meyerowitz et al., 1989). Analysis of the floral homeotic mutants of two different plant species *Arabidopsis thaliana* (Thale cress, henceforth called *Arabidopsis*) (Bowman et al., 1989, 1991a; Yanofsky et al., 1990), and *Antirrhinum majus* (Snapdragon, henceforth called *Antirrhinum*) (Schwarz-Sommer et al., 1990; Sommer et al., 1990) for over twenty years was of great value for resolving the general mechanisms of floral development (Coen & Meyerowitz, 1991; Theißen et al., 2000; Krizek and Fletcher, 2005).

1.2 Developmental genetics of floral homeotic mutants

Of the plant examples showing homeotic changes the floral homeotic mutants are particularly well known. In flowers of homeotic mutants more or less perfectly developed organs occur at sites, where floral organs of another type would normally occur.

A vast majority of flowers in the plant kingdom consist of four different circular organ whorls, resembling part of the segmental structures in plants. Though all of the whorls can be multiplied, reduced or transformed into each other, the basic number of whorls in eudicots remains to be four. In the first and outmost whorl sepals are typically present, followed by petals in the neighbouring second whorl. These two outer whorls establish the perianth, in which first whorl sepals protect inner floral organs and typically second whorl petals attract pollinators. The reproductive organs of a flower are nested in the inner two whorls with stamens in the third floral whorl and a single carpel or several separated or fused carpels in the innermost fourth whorl. Floral homeotic mutants are categorised into three different classes, called A, B and C (Figure 1A-C). In A-class mutants the typical organs of the first two floral whorls are replaced by carpels in the first and stamens in the second whorl. In B-class mutants organs of the second and third whorl are replaced by sepals and carpels, respectively, and in C-class mutants the reproductive organs of the two innermost whorls are replaced by petals in the third whorl and reiterating floral perianth organs in the central fourth whorl (Meyerowitz et al., 1989). The reiteration of perianth organs in the fourth whorl, the well-known phenomenon of ´filled flowers`, is caused by the loss of determinacy resulting in the formation of additional flowers in the flower (Meyerowitz et al., 1989). Three different floral homeotic functions are proposed to explain how the unique identities of floral organs are established during development. They are fused into a simple combinatorial genetic model, the ABC-model (Figure 1F). The functional classes are termed A, B and C, corresponding to the aforementioned mutant classes. Hence A function specifies sepals, A- and B-function together specify petals, B- and C- function together specify stamens and the C-function alone specifies carpel identity (reviewed by Coen & Meyerowitz, 1991; Theißen 2001a, b, Ferrario et al 2004, Krizek and Fletcher, 2005). After development of the ABC model some shortcomings became apparent, e.g. the A, B and C genes are indeed required but not sufficient for specification of floral organ identity. Analysis of floral homeotic mutants also helped closing this gap through identification of additional class D and E genes. Class D genes are involved in ovule development (Angenent and Colombo, 1996), whereas class E gene function added to the other A, B and C-class combinations is both required and sufficient to specify identity in floral organs. The respective E-class mutant phenotype (see Figure 1E) shows transformation of the typical floral organs into leaf-like organs (Pelaz et al., 2000; Ditta et al., 2004). As a result of the identification of additional organ identity gene classes the existing model was extended to an ABCDE model. In parallel to genetic analyses of homeotic mutants, transgenic studies revealed that the combinatorial function of the genes is accomplished by complex formation between proteins encoded by the different gene classes (Honma and Goto, 2001). This formation of multimeric complexes provided an explained how the combinatorial function of the floral identity genes is established at the molecular level. Formation of tetrameric protein complexes which consist of at least one E-class protein and one or two types of ABC-class proteins was described in the ´floral quartet model`. This model has later been extended to the determination of ovules involving class D genes (Theißen, 2001b; Theißen and Saedler, 2001, Melzer et al. 2006). Henceforth all following considerations will use the simpler ABC model, as the differences in floral organ identity in floral homeotic mutants mostly result from changes in these gene classes.

The organ identity genes in *Arabidopsis thaliana* are *APETALA1* (*AP1*) and *APETALA2* (*AP2*) in case of class A function, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in case of class B function, and *AGAMOUS* (*AG*) in case of class C function. They are all encoding transcription factors as reviewed by Theißen (2001a) and by Krizek and Fletcher (2005). Hence, as transcription factors the protein products of these ABC genes regulate the transcription of target genes downstream in the developmental cascadewhich finally control the differentiation and growth of the different floral organs. Except for *AP2*, all ABC genes are members of the MADS-box gene family. This gene family is characterised by a conserved sequence coding for a special DNA binding domain. This sequence element was named MADS-box after the founding members of this new gene family with the yeast transcription factor *<u>M</u>INICHROMOSOME MAINTENANCE 1* (*MCM1*), *<u>A</u>GAMOUS* (*AG*) and *<u>D</u>EFICIENS A* (*DEF A*) of *Arabidopsis* and *Antirrhinum*, respectively, and the *<u>S</u>ERUM RESPONSE FACTOR* (*SRF*) of mammals (Schwarz-Sommer et al., 1990; Riech-mann and Meyerowitz, 1997). The similarity with yeast and animal MADS-box genes and the ability of plant MADS-box genes to bind to DNA supported their function as transcription factors. The *AP2* gene is the only exception in this case, as it belongs to the EREBP-transcription factor family (Jofuku et al., 1994, Bomblies et al., 1999). The name is derived from the abbreviations of the consecutive protein domains from N-terminal to the

C-terminal end: MADS-, Intervening-, Keratin-like and C-terminal-domain (Theißen et al., 2000).

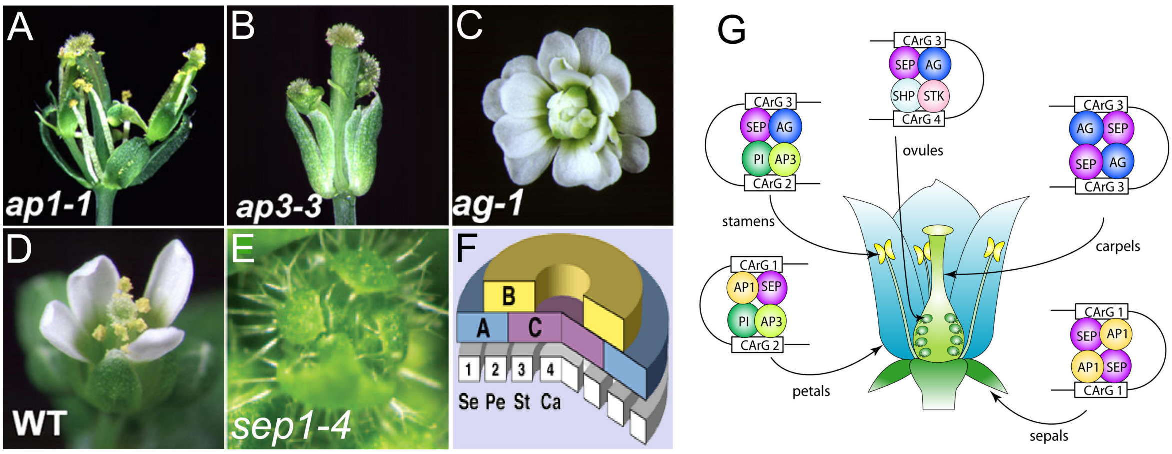

Figure 1. Homeotic mutants and wild-type flower of *Arabidopsis thaliana* and models explaining the interactions of floral homeotic proteins. (**A**) class A mutant with petals replaced by stamens and sepals replaced by carpels; (**B**) class B mutant with stamens replaced by carpels and petals replaced by sepals; (**C**) class C mutant with stamens replaced by petals, carpels replaced by sepals and loss of determinacy; (**D**) wild-type flower; (**E**) class E quadruple mutant with all floral organs replaced by leaves, loss of whorl arrangement and determinacy; (**F**) simple ABC model explaining combinatorial gene function in floral organ determination; (**G**) floral quartet model explaining formation of tetrameric complexes which bind to target gene motifs (CArG-boxes). (Pictures A-D and F from Riechmann and Meyerowitz, 1997; E from Krizek and Fletcher 2005; G from Melzer et al., 2006)

1.3 The role of homeotic mutants in the evolution of flowers

In addition to the important role that the floral homeotic mutants of *Arabidopsis* or *Antirrhinum* played in the elucidation of the developmental control of organ identity, the advantage of using such model systems for the analysis of their evolutionary potential might be in principle imaginable, but exactly in these cases the mutant phenotypes are either completely sterile or at least severely hampered in propagation. The potential of mutant analysis is based on the combinatorial function of the organ identity genes, which may, for example, enable switches of organ positions to other whorls by simply changing or extending their expression profiles towards the respective whorls through a single mutational event. This may occur during mutagenesis experiments in vitro and upon ´natural` mutation. In case of a ´natural` mutation the resulting novel flower structure, e.g. a homeotic transformation, is exposed natural evolution and may proof itself as either deleterious, neutral or even beneficial, leading to loss or fixation of an respective allele. In a number of plant families beneficial modifications of organ identity are obviously well established. In these cases homeosis is caused by changes in expression profiles of organ

identity genes as shown exemplarily for members of the Lily-family as well as in the Ranunculaceae. Here, the outmost perianth whorl organs resemble third whorl petal organs in colour and form. Those shifts in floral structure are caused by extended expression of class B genes in the outer floral whorl (Kanno et al. 2003, Kramer et al. 2007), demonstrating how differential regulation of single organ identity genes controls distinct floral architectures. Hence, investigations on the role of developmental control genes were more often integrated in evolutionary questions about morphological diversification in evolution and inspired the new biological field of evolutionary developmental biology (´evo-devo`) (for details see Theißen et al., 2000; Carroll, 2001; Arthur, 2002).
Despite those studies explained above, where morphological diversity based on homeotic changes has already been evolved and well established over several million years, it is still controversially debated if and how homeotic conversions played a role in initial processes of diversification (Theißen, 2006). Concerning these questions the well known floral homeotic mutants of *Arabidopsis* and *Antirrhinum* are of little help, because all of them are affected in reproduction and are not able or at least hampered to compete with wild-type plants in natural environments. A prominent example is the typical class C loss-of-function mutant, which completely lacks reproductive organs (Yanofsky et al., 1990). Interestingly, no homeotic mutants that occur in stable natural populations are known from *Arabidopsis* so far, despite decades of intensive (field) research. Therefore recently emerged naturally occurring floral mutants have to be found, which show homeotic conversions that do not hamper reproductive function and which can be tested rigorously for their performance in wild populations. Analysis of those kinds of homeotic mutants will consequentially reveal the real potential of homeotic mutants in establishing morphological innovations in flowers (Theißen, 2000; Bateman and DiMichele, 2002).
A detailed review on the aspects of homeotic conversions and their role in evolution of plant (flower) structure are covered in Manuscript I (Nutt et al., 2006) and in Hintz et al., (2006).

1.4 A floral homeotic variant of *C. bursa-pastoris* helps investigating the evolutionary role of homeosis in plants

A number of homeotic mutants were reported in historical and more recent publications (reviewed in Gottschalk, 1971; Meyerowitz et al., 1989; Ronse de Craene, 2003). Among them are several naturally occurring ones, however, information on the evolutionary potential of these natural mutants is scarce. In a few more detailed historical and current analyses a number of obstacles were encountered that either limited the extent of investigations, or even made a comprehensive analysis impractical. Reasons for this were, for instance, restricted propagation of the mutant due to reduced plant fitness, lack of heritability or instability of inheritance, very long generation times of the affected plant species or obstacles with the application of molecular tools. Two prominent examples are the *bicalyx* mutant of the perennial shrub *Clarkia concinna*, where petals are replaced by sepals (Ford und Gottlieb, 1992) and a supposedly clonal flock of a peloric *Linaria vulgaris* where bilateral flower symmetry is changed to radial symmetry (Cubas et al., 1999). Although it was shown that both of these mutant phenotypes are caused by a change in a single genetic locus, these two case studies encountered several of the above mentioned problems, which disqualified the mutants as useful models to study natural evolution both molecularly and in the field, as reviewed in more detail in Manuscript I (Nutt et al., 2006) and Hintz et al., (2006).

The problem to find a suitable model system for the above mentioned combined analysis remained virulent until a population of the floral homeotic variant of *Capsella bursa-pastoris* was discovered 1991 by Reichert (1998). In this variant flowers display a homeotic transformation of petals into stamens in the second floral organ whorl. This phenomenon in *C. bursa-pastoris* was first described by Opitz (1821) and termed decandric, because of the increased number of stamens to ten, rather then the typical number of six. Opitz even described the variety as a new species, *Capsella apetala*. During the centuries the occurrence of this variant was reported from several habitats in Europe and was repeatedly discussed in the historical botanic literature, as reviewed in more detail in manuscript I (Nutt et al., 2006). Reichert (1998) reported a natural population of a decandric variant of *C. bursa-pastoris* in Rheinhessen, Germany. He reported the stability of the phenotype over several years even though it grows mixed with wild-typic plants in one population in a ruderal habitat plunging a vine-yard landscape. At the beginning of the work described here, another population of decandric *C. bursa-pastoris* was discovered in Westfalia (Germany) near Warburg at the Desenberg. Members of both populations were cultivated and after initial preliminary analysis introduced as a new model system for the analysis of this syndrome in a combined and, as possible, complete way in morphological, genetic, molecular and ecological terms in manuscript I (Nutt et al., 2006) and in Hintz et al. (2006). In this publication the decandric syndrome is termed *Stamenoid petals* (*Spe*) due to its phenotype and co-dominant mode of inheritance. Furthermore a hypothesis is presented that explains the organ transformation in the second floral whorl. We suggest an extension of the expression domain of a class C organ identity gene from the inner two whorls to the second whorl that changes organ specification, assuming that the rules of the ABC model can be applied to wild-type flowers of *C. bursa-pastoris* in the same manner as in flowers of *Arabidopsis* (Figure 2).

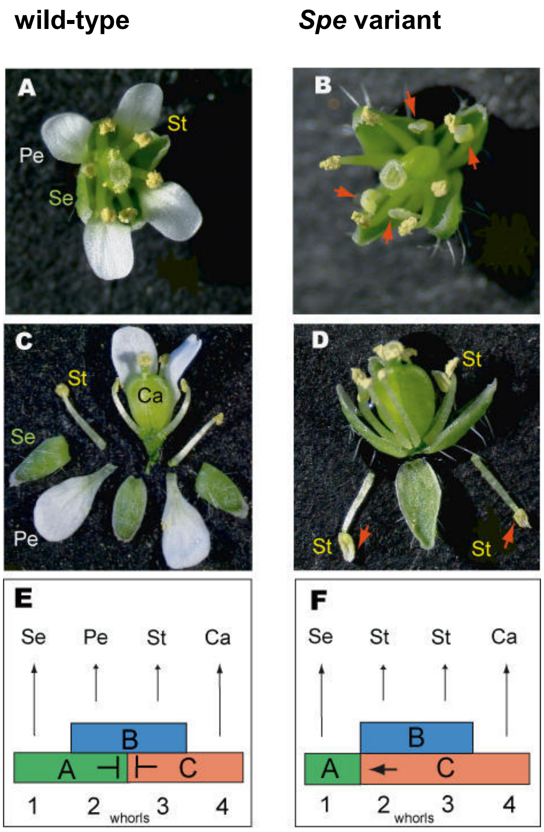

Figure 2. Floral structures of *Capsella bursa-pastoris* wild-type (**A**, **C**) and *Spe* (**B**, **D**) plants and hypothesised organ identity determination (**E**, **F**) assigned from the ABC model.
A, Typical wild-type floral habitus with four petals and six stamens; **C**, dissected wild-type flower.
B, Habitus of typical decandric structure of *Spe* flowers with four additional stamens instead of petals; **D**, dissected *Spe* flower.
E, Assumed ABC model of the wild-type, similar to *Arabidopsis*; **F**, assumed modification of ABC model by extension of Class C gene expression in the second floral whorl in the *Spe* variant.

Abbreviations: Ca, carpels; Pe, petals; Se, sepals; St, stamens. Red arrows pointing to transformed organs of second whorl. (Figure taken from Nutt et al., 2006)

1.5 *Capsella bursa-pastoris* as a model species

The following aspects characterise the species *C. bursa-pastoris* in general and demonstrate additional vantages that arise from its establishment as a model plant. *C. bursa-pastoris* (L.) Medik. is well known with its common name Shepherd`s purse, referring to its characteristic heart-shaped fruits. Within the family of Brassicaceae it is a member of a small genus, which is very closely related to the genus *Arabidopsis* with an estimated divergence time of about 10 million years (Koch et al., 2001). The genus comprises most probably only three species which, however, differ remarkably in habitat range, breeding systems and ploidy level (Hurka et al., 2005). The tetraploid *C. bursa-pastoris* is predominantly selfing, grows in ruderal habitats almost all over the world and represents one of the most widespread flowering plants on earth (Hurka et al., 2003). The other *Capsella* species, *C. grandiflora* (self-incompatible and therefore obligately outbreeding) and *C. rubella* (self-fertile) are diploid and show more restricted distributions. The relationship within the three species is not satisfyingly resolved yet, but the long held notion of the origin of *C. bursa-pastoris* by auto-or alloploidisation of the other genus members (Hurka et al., 1989; Hurka and Neuffer, 1997) was recently refuted by Slotte et al. (2006). However, the character states described for *C. bursa-pastoris* seem to represent the most derived ones in this genus (Hurka et al., 2005).

C. bursa-pastoris has already been a classical model organism for description of early development in embryos of eudicot plants (Schulz and Jensen, 1968). It has also been characterised by its variability of morphological traits such as flower, fruit and leaf shape as well as variability of quantitative traits. A number of biogeographic studies about colonisation processes have been reported so far as well (Hurka et al., 1989 and 2003; Neuffer and Hurka, 1999; Linde et al., 2001). In the last decades a vast number of genomic and molecular tools have been developed for *Arabidopsis*, some of which have already been utilized in *Capsella* species (a.o. Boivin et al., 2004). The genus therefore offers an excellent opportunity not only to study morphogenetic phenomena like the *Spe* variant, but also to investigate polyploid speciation and the genetic basis of quantitative traits in a wild polyploid species without the influence of generations of artificial man-made breeding effects (Slotte, 2007). It may also offer an opportunity to complement the pitfalls of the ´streamlined genomes` with low ploidy level and small genome sizes of well investigated model plants such as *Arabidopsis*. In this thesis, this opportunity is provided by the *Spe* syndrome, where petals are replaced by stamens, which is known from several plant species as reviewed in manuscript I (Nutt et al., 2006), but not known from any of the intensively investigated ecotypes of *Arabidopsis* so far. Clarifying the molecular mechanisms of phenotype development in *Spe* plants will shed light on still enigmatic developmental processes of cadstral regulation of organ identity genes in flowers.

1.6 Aims of this work

The importance of homeosis on the evolution of novelties is still a controversially debated topic. Therefore, naturally occurring floral homeotic mutants based on a single altered locus and occurring in natural populations need to be established as model systems to give the opportunity to study the role of homeosis in flower evolution in a comprehensive way morphologically, genetically molecularly and ecologically. However, in the known and analysed cases of homeosis in plants the mutants are seriously hampered in reproduction like the well known homeotic mutants of *Arabidopsis* and *Antirrhinum*. In the cases where natural occurrence and heratibility has been shown, the underlying molecular mechanisms are not understood yet, like in *Clarkia concinna* (Nutt et al., 2006, Ford and Gottlieb, 1992). Consequentially the central objective of this thesis was to identify a model system that facilitates investigations in all fields of interest and to establish this system in a detailed combined morphological genetic and molecular analysis.

Within the scope of this analysis three major aims were pursued:

The intention of the initial phase of the project was to compile already known facts about the role of homeosis in a concomitant review article, to display the potentials of *C. bursa-pastoris* as a model species and to introduce the *Spe* variant, which shows a presumably perfect homeotic change in a natural mutant. We also developed a hypothesis for the molecular cause of the Spe phenotype based on ectopic expression of a class C organ identity gene and provided an experimental program to study this phenomenon as detailed as possible and to test the above mentioned hypothesis. (**manuscript I**).

The central aim of this thesis was the detailed morphological, genetic and molecular analysis of the *Spe* variant, with the following central questions: (1) Do we find a case of complete homeosis in *Spe,* in which the identity of one organ type is completely changed into another identity morphologically and functionally and does the *Spe* phenotype show any pleiotropic effects? (2) Is the mutant phenotype stably inherited and does the mode of inheritance allow conclusions about the underlying molecular mechanism? (3) Can ectopic expression of an *AGAMOUS*-like gene be observed in the transformed organs, as it would be expected considering the determination of stamen identity known from the ABC model in *Arabidopsis*? Another goal arose from the latter question, namely if the *Spe* locus is linked to one of the *AG*-like genes and represents therefore a mutated allele of the respective gene. Which molecular mechanisms cause the changed function of the *Spe* locus? This is an important piece of the PhD thesis of Janine Ziermann, that completed my results in the submitted manuscript (II). In **manuscript II** the results of the investigations are reported in detail. I showed that the *Spe* variety displays an (almost) perfect homeotic shift from petals to stamens that is driven by a single co-dominant allele in two different *Spe* variant-lines. The mode of inheritance already showed a dose (allele)-dependent effect in *Spe* suggesting a gain-of-function mutation in the regulatory region of a single affected gene. I also showed that ectopic expression of two *AG*-like genes, *CbpAG* and *CbpSTK*, correlate the organ transformation. Further results of J. Ziermann showed that it is very likely a change in a candidate *cis*-regulatory region of the *CbpAGa* gene that might be responsible for the deviating expression patterns and the resulting organ transformation.

For the completion of any other molecular genetic analysis in *C. bursa-pastoris*, germline transformation capability is an essential prerequisite, as this opportunity opens the door for efficient applications such as specific knock-down of genes of interest as well as large scale insertion mutagenesis experiments in functional studies. Consequentially, another aim of this project was to publish a transformation protocol (**manuscript III**), established by C. Bartholmes during her diploma thesis mostly under my supervision, to provide the growing community of groups working with *C. bursa-pastoris* with this important tool.

2 Overview of the manuscripts

I. **P. Nutt, J. Ziermann, M. Hintz, B. Neuffer, and G. Theißen (2006): *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants. *Plant Systematics and Evolution* 259, pp 217-235.**

We report specific taxonomic, ecological and genetic vantages which qualify the natural homeotic variant *Stamenoid petals* (*Spe*) of *C. bursa-pastoris* to study the impact of floral homeotic mutants for the evolution of floral structures and described a detailed experimental program for the analysis of *Spe*. Additionally, the publication gives an overview of the current discussion about the benefits of using homeotic mutants to study macroevolutionary steps in general and in particular for flower evolution.

I collected part of the literature, wrote the first draft and prepared the figures of the manuscript. Janine Ziermann wrote the field study part of the chapter "studying *Spe*" and provided the respective material for Figure 4, Maren Hintz and Barbara Neuffer corrected and completed the manuscript in terms of evolutionary importance of homeosis and *Capsella* phylogeny. Günter Theißen corrected, completed and improved the whole manuscript.

II. **P. Nutt[1], Janine Ziermann[1] and G. Theißen (submitted 7th of May 2008 to *The Plant Cell*) Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural homeotic floral variant of *Capsella bursa-pastoris.***
([1] These authors contributed equally to this work)

In this manuscript we reported on the combined morphological, genetic and molecular analysis of the floral homeotic variant *Spe* in *C. bursa-pastoris* in which petals are transformed into stamens. We demonstrated that the *Spe* phenotype shows a perfect homeotic transformation without pleiotropic effects, caused by the same single co-dominant locus in two different *Spe* populations. We also demonstrated co-segregation of only the *CbpAGa* gene locus (out of other related candidate genes) with the *Spe* phenotype and ectopic expression of the same gene in the transformed organs, suggesting that *Spe* is a mutant allele of *CbpAGa*. We identified a candidate negative *cis*-regulatory element in the *CbpAGa* gene that is deleted in *Spe* and is therefore likely responsible for the *Spe* syndrome.

For this publication I performed all morphological and genetic analyses, the isolation of cDNA sequences, all Southern and *in situ* hybridisation experiments as well as the phylogeny reconstruction. I wrote the parts of the corresponding manuscript chapters and (together with J. Ziermann) the first draft of the discussion and I prepared the Figures (Figure 6 and Suppl. Figure 4 together with J. Ziermann). Janine Ziermann performed the isolation of genomic sequences, the identification of SNPs, the co-segregation analysis via pyrosequencing and identified the candidate deletion in the *CbpAGa* gene. She prepared the corresponding chapters and Figures in the manuscript. Günter Theißen wrote the introduction and corrected, completed and improved the whole manuscript.

III. C. Bartholmes, P. Nutt and G. Theißen (2008) Germline transformation of Shepherd's purse (*Capsella bursa-pastoris*) by the 'floral dip' method as a tool for evolutionary and developmental biology. *Gene* 409, pp 11-19.

In this publication we reported on an adapted ´floral dip` germline transformation protocol for *Capsella bursa-pastoris* and recommended its usage as a tool for evolutionary and developmental biology. We described the verification of transformation, expression, stable integration and inheritance of the transgene in the plant genome by usage of marker genes and Southern hybridisation. The most efficient *Agrobacterium* strain and composition of infection media was identified.

Conny Bartholmes did all experimental work in the context of her diploma thesis and wrote the ´Materials and Methods` chapter, a short summary of the results and first drafts of the discussion, and prepared Figure 2. I supervised the lab experiments, corrected, completed and improved the manuscript and prepared all Tables and Figures. Günter Theißen inspired and supervised the diploma work. He corrected and improved the manuscript.

3 Manuscript I

P. Nutt, J. Ziermann, M. Hintz, B. Neuffer, and G. Theißen (2006): *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants. *Plant Systematics and Evolution* 259, pp 217-235.

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Capsella as a model system to study the evolutionary relevance of floral homeotic mutants

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Abstract. Several lines of evidence suggest that homeotic changes played a considerable role during the evolution of flowers. This, however, is difficult to reconcile with the predominant evolutionary theory which rejects any drastic, saltational change of the phenotype as reasonable mode of evolution due to its assumed negative impact on the fitness of the affected organism. A better understanding of the evolutionary potential of homeotic transitions requires a study of the performance of respective mutant varieties in the wild. Here we introduce "*Stamenoid petals*" (Spe), a variety of *Capsella bursa-pastoris* (shepherd's purse), as a suitable model to study the evolutionary potential of floral homeotic mutants. In the flowers of the *Spe* variety all petals are transformed into stamens, while all other floral organs are unaffected. In contrast to most other homeotic mutants the *Spe* variety occurs on several locations in relatively large and stable populations in the wild. Due to its close relationship to the model plant *Arabidopsis thaliana*, the *Spe* variety of *C. bursa-pastoris* can be rigorously studied, from the molecular genetic basis of the phenotype to its consequences on the fitness in wild habitats. Investigations on *Spe* may thus help to clarify whether homeotic transformations have the potential to contribute to macroevolution.

Key words: ABC model, *Capsella bursa-pastoris*, flower development, homeosis, macroevolution, MADS-box genes, stamenoid petals.

Capsella as a model system to study the evolution of flower development

Capsella is a small genus within the mustard family (Brassicaceae). According to Hurka and co-workers it comprises only three species, which, however, show remarkable differences in ploidy level, breeding systems and habitat range (Hurka and Neuffer 1997, Zunk et al. 1999, Hurka et al. 2005). Two of the species, *Capsella grandiflora* and *Capsella rubella*, are diploid, while the third one, the well-known weed *Capsella bursa-pastoris* (shepherd's purse) is tetraploid. *C. grandiflora* is obligately outbreeding due to a sporophytic self-incompatibility (SI) system and grows in a very limited habitat in western Greece, Albania and northern Italy. Compared to the other two species it has relatively large, fragrant flowers with showy petals, obviously to attract pollinators. In contrast, *C. rubella* is a completely selfing plant with comparatively small flowers that grows around the Mediterranean Sea (Hurka et al. 1989). The predominantly selfing *C. bursa-pastoris* is distributed in ruderal habitats all over the world, except in the tropics and subtropics (Hurka and Neuffer 1997). In fact, together with *Polygonum aviculare*, *Stellaria media*, *Poa annua* and *Chenopodium album*, *C. bursa-pastoris* belongs to the most wide-spread flowering plants on our planet (Coquillat 1951, Hurka et al. 2003).

The relationships between the three *Capsella* species have not been resolved yet. It has been suggested that *C. bursa-pastoris* originated by autopolyploidy within *C. grandiflora*, or by allopolyploidy between *C. rubella* and an unknown ancestor (Hurka and Neuffer 1997; Slotte et al., unpubl. data). Some recent molecular data suggest that *C. grandiflora* and *C. rubella* are sister species to the exclusion of *C. bursa-pastoris* (Slotte et al., unpubl. data), suggesting that autopolyploidy in a diploid common ancestor of all three species gave rise to the tetraploid *C. bursa-pastoris* lineage.

Anyway, comparison with outgroup species strongly suggests that concerning reproductive biology *C. grandiflora* represents mostly ancestral and *C. bursa-pastoris* mostly derived character states. For example, the self-compatible (SC) breeding system of *C. rubella* and *C. bursa-pastoris* was evidently caused by breakdown of the self-incompatibility (SI) system still active in *C. grandiflora* (Hurka and Neuffer 1997, Hurka et al. 2005). By enabling reproduction via selfing and thus reproductive assurance under circumstances of poor outcrossing possibilities when mates (or pollinators) are scarce (Shimizu et al. 2004, Shimizu and Purugganan 2005, and references cited therein), this has quite certainly contributed to the colonisation potential of *C. rubella* and *C. bursa-pastoris*. The latter species may have further boosted its colonisation potential and weediness due to polyploidisation followed by a shift to disomic inheritance, as revealed e.g. by the inheritance of allozymes (Hurka et al. 1989, Hurka and Düring 1994), which led to 'fixed heterozygosity' and thus increased the intraspecific genetic diversity. This may have helped to avoid inbreeding depression when outcrossing is rare; outcrossing rates in the field of 0–20 % have been determined and may strongly depend on environmental conditions such as the weather (Hurka and Neuffer 1997). While self-incompatible species may use large petals and floral scent to attract pollinator insects, pollinator attraction may have become less important in the species with SC breeding system, which may explain the reduction of floral size and the loss of floral fragancy in both *C. rubella* and *C. bursa-pastoris*.

Within the small genus *Capsella* we can thus follow up the transition from a diploid, self-incompatible, obligatory outcrossing species with comparatively large and attractive flowers but a quite restricted area of distribution (*C. grandiflora*), to a tetraploid, self-compatible, predominantly selfing species with relatively small flowers but a breathtaking colonisation success almost all around the globe (*C. bursa-pastoris*) (Paetsch et al., unpubl. data). The dramatic difference in 'invasiveness' between *C. grandiflora* and *C. bursa-pastoris* is quite remarkable, and needless to say that the how's and why's of this difference are of great evolutionary and agricultural interest (Hurka et al. 2003).

However, most aspects of the evolutionary transition within *Capsella* are also found in other plant groups, including close relatives of *Capsella*. For example, the shift from outcrossing to self-pollination is one of the most prevalent evolutionary transitions in flowering plants (Stebbins 1950, Barrett 2002, Mable 2005).

Changes from self-incompatible, outcrossing species with complete and conspicuous flowers to self-compatible, selfing species with small or reduced flowers have occurred many times even within the evolution of the Brassicaceae alone. A good example is the genus *Arabidopsis*, including the major plant model *A. thaliana*. Like *C. bursa-pastoris*, *A. thaliana* is a weedy, predominantly selfing species with a reported outcrossing rate of about 1% that prefers disturbed, man-made habitats (reviewed by Hoffmann et al. 2003). The lineage that led to extant *A. thaliana* separated roughly about 5–6 million years ago from the *Arabidopsis lyrata* lineage, which has still an ancestral SI system and hence is an outcrossing species.

Even more radical evolutionary changes can be observed in *Lepidium*, another genus of Brassicaceae (Bowman 2006). While basal lineages within *Lepidium* are diploids and generally have the canonical Brassicaceae floral ground plan (see below), derived lineages are often self-fertilizing allopolyploids with reduced floral structures and great colonizing potentials. Also in case of *Lepidium* allopolyploidy is assumed to increase the gene pool to avoid inbreeding depression (Lee et al. 2002, Bowman 2006). More than half of the about 200 *Lepidium* species have only two or four rather than six stamens, and in most of these species, petals are rudimentary (Bowman 2006). Thus *Lepidium* species show remarkable simplifications of floral structure beyond simple reduction in floral size.

Obviously, transitions from diploid, self-incompatible, obligatory outcrossing species with comparatively large and attractive flowers, to polyploid, self-compatible, predominantly selfing species with floral reductions, represent a frequent trend in the evolution of Brassicaceae. *Capsella* might thus serve well in comparative studies aiming at understanding parallel and convergent evolution of floral features.

In addition, the genus *Capsella* shows a very rare phenomenon which, nevertheless, could be of great evolutionary importance, i.e. the occurrence of a homeotic variety in quite stable populations in the wild. Several lines of evidence suggest that homeotic changes played a considerable role during the evolution of flowers, but the relevance of homeotic transformations during the origin of morphological novelties has remained a very controversial topic (reviewed in Theißen 2006). How floral homeotic varieties are established in natural populations has remained almost completely unknown so far (Theißen 2000). Due to its close relationship to the model plant *Arabidopsis* numerous experimental tools are available to study the genus *Capsella*, and more are being developed. *Capsella* hence provides an unprecedented opportunity to investigate the origin, performance and evolutionary potential of a floral homeotic mutant in nature.

Floral homeotic mutants and floral organ identity

To fully understand the scientific relevance of the homeotic *Capsella* variety the meaning of 'homeosis' has first to be clarified. The term was coined by William Bateson in 1894 to describe a type of variation in which 'something has been changed into the likeness of something else'; a well-known example is provided by the *Antennapedia* mutant of the fruit fly *Drosophila melanogaster* which has antennae replaced by leg-like organs (Lewis 1994).

Homeotic mutants are a quite frequent phenomenon in plants, where both vegetative and reproductive organs can be affected (Sattler 1988, Meyerowitz et al. 1989). Especially well-known are floral homeotic mutants, i.e. mutant plants with flowers that have more or less normal floral organs in places where organs of another type are typically found. The analysis of such mutants has been of great help in understanding as to how the different floral organs acquire their specific identity during flower development (Ferrario et al. 2004, Krizek and Fletcher 2005).

The flowers of most Brassicaceae have a well conserved body plan that applies also to the major model plant, *Arabidopsis thaliana*, as well as all *Capsella* species. It comprises four different classes of organs arranged in four whorls, i.e. four sepals in the first, outermost whorl; four petals in the second whorl; six stamens in the third whorl; and two fused carpels in the fourth and innermost whorl (Fig. 1D). In homeotic mutants the identity of floral organs is changed in a systematic way (Coen and Meyerowitz 1991). In *Arabidopsis thaliana* such mutants come in three major

Fig. 1. Flower phenotypes of loss-of-function mutants in floral homeotic genes of *Arabidopsis thaliana*. **A** *ap1-1* is a class A mutant affected in the *APETALA1* (*AP1*) gene that has sepals transformed into carpelloid organs and petals transformed into stamenoid organs. **B** *ap3-3* is a class B mutant affected in the *APETALA3* (*AP3*) gene that has petals transformed into sepaloid organs and stamens transformed into carpelloid organs. **C** *ag-1* is a class C mutant in the *AGAMOUS* (*AG*) gene that has stamens transformed into petaloid organs; carpels are replaced by another mutant flower due to the loss of determinate growth of the floral shoot, so that the number of floral organs is significantly increased in the 'filled flowers' of the mutant. **D** Wild-type flower of *A. thaliana*. Figures from Riechmann and Meyerowitz (1997), with kind permission from Walter de Gruyter GmbH & Co. KG, Berlin, Germany

classes, termed A, B and C. Ideal class A mutants have carpels in the first whorl instead of sepals, and stamens in the second whorl instead of petals (Fig. 1A). Class B mutants have sepals rather than petals in the second whorl, and carpels rather than stamens in the third whorl (Fig. 1B). Class C mutants have petals instead of stamens in the third whorl, and replacement of the carpels in the fourth whorl by sepals. In addition, flower development in these mutants is indeterminate, i.e. there is continued production of mutant floral organs inside the 4th whorl (Fig. 1C). This results in the characteristic phenotype of 'filled flowers' which is well known from many wild and ornamental plants, including *Antirrhinum*, *Rosa* (rose), *Prunus* (e.g. cherry), *Petunia* and *Tulipa* (tulip).

The existence of these classes of mutants suggested that development of the flower is sculpted by homeotic selector genes ('floral organ identity genes') whose expression gives the different floral organs their identity. Such genes can be considered as acting as major developmental switches that activate the entire genetic program for a particular organ, and repress all genes that would interfere with proper organ development. The 'ABC model' was proposed to explain how homeotic genes control floral organ identity (Coen and Meyerowitz 1991). It maintains that organ identity in each whorl is determined by a unique combination of three organ identity gene activities, called A, B and C (Fig. 2E). Expression of A alone specifies sepal formation. The combination A + B specifies the development of petals, and B + C specifies the formation of stamens. Expression of C alone determines the development of carpels. In order to explain the three classes of floral homeotic mutants, the ABC model proposes that the class A and class C genes negatively regulate each other, so that the class A genes become expressed throughout the flower when the class C gene is defect, and *vice versa* (for reviews of the ABC model, see Theißen 2001a, Ferrario et al. 2004, Krizek and Fletcher 2005).

In *Arabidopsis* the class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the class C gene by *AGAMOUS* (*AG*). Molecular cloning of these genes revealed that they all encode putative transcription factors (for a review, see Theißen 2001a, Krizek and Fletcher 2005). Thus the products of the ABC genes probably

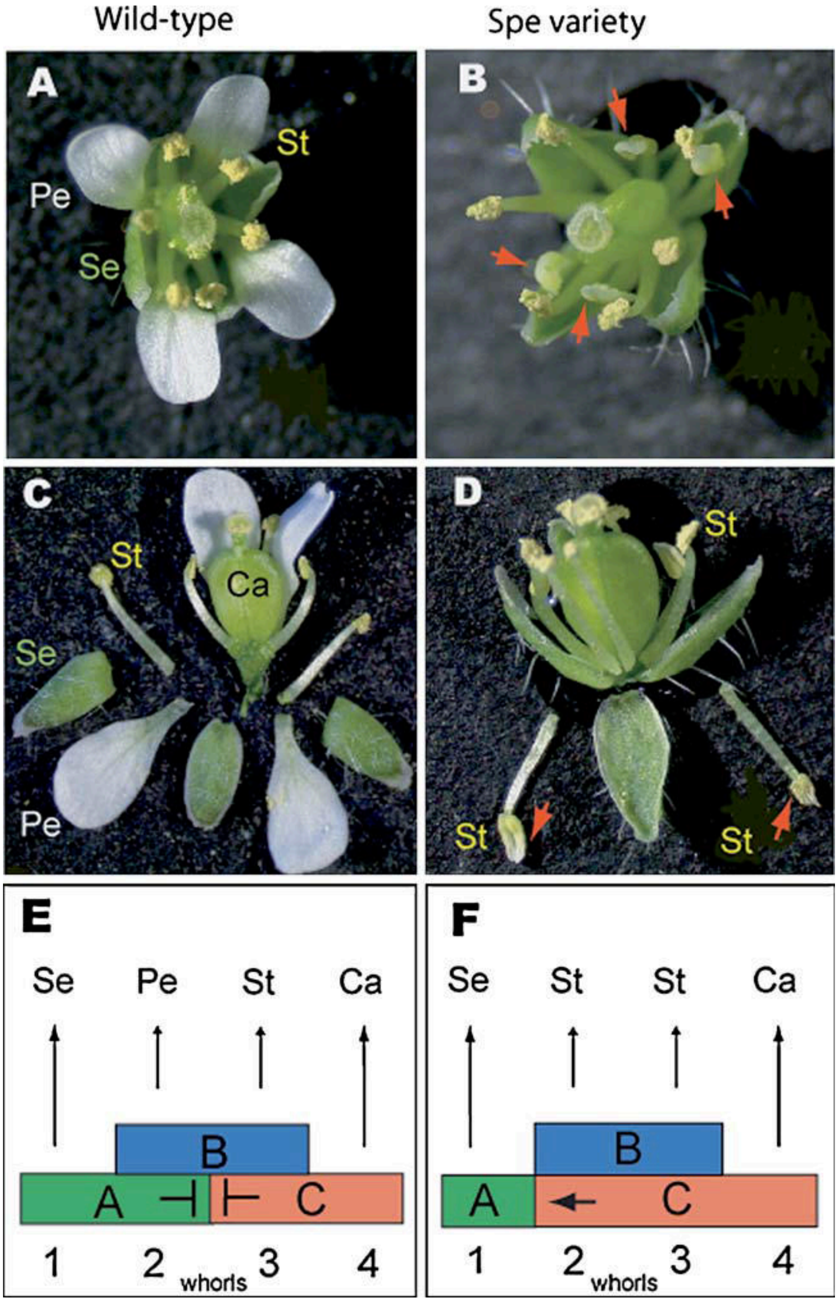

Fig. 2. Structures of flowers of *Capsella bursa-pastoris*, and explanations of organ identities by the ABC model. **A, C** wild-type flowers, with 4 green, leaf-like sepals in the first, outermost floral whorl, 4 white and showy petals in the second whorl, 6 stamens in the third whorl, and two fused carpels in the fourth, innermost whorl. **B, D** Flowers of the *Spe* variety, which have the same structure as wild-type flowers, except that petals in the second whorl are transformed into stamens (marked by red arrows). **A, B** Flower habitus; **C, D** Partially dissected flowers, to better demonstrate organ identity. **E** Classical ABC model explaining organ identity in wild-type flowers of *Capsella* and *Arabidopsis*. **F** Modified ABC model for the *Spe* variety of *C. bursa-pastoris*. Abbreviations: *Ca*, carpels; *Pe*, petals; *Se*, sepals, *St*, stamens

all control the transcription of other genes ('target genes') whose products are directly or indirectly involved in the formation or function of these organs. Except for *AP2* all ABC genes are MADS-box genes encoding MADS-domain transcription factors (for reviews about MADS-box genes in plants, see Becker and Theißen 2003, De Bodt et al. 2003, Riechmann and Meyerowitz 1997, Theißen et al. 2000).

It soon turned out that the ABC genes are required, but not sufficient for the specification of floral organ identity. Moreover, the ABC model did not provide a molecular mechanism for the interaction of floral homeotic genes during the specification of floral organ identity (Theißen 2001b). These shortcomings of the ABC model could meanwhile be overcome. Identification of class D genes involved in ovule development, and class E genes required for development of sepals, petals, stamens and carpels, led to the extension of the ABC model to the 'ABCDE' model (Angenent and Colombo 1996, Pelaz et al. 2000, Theißen 2001a, Ditta et al. 2004). Transgenic studies in *Arabidopsis* revealed that the ABCDE genes are not only required, but are even sufficient at least to specify petal and stamen identity (Honma and Goto 2001). Moreover, the interaction of floral homeotic genes could be explained at the molecular level by the capacity of floral homeotic proteins to form tetrameric complexes of transcription factors (Egea-Cortines et al. 1999, Honma and Goto 2001), as outlined by the 'floral quartet model' (Theißen 2001a, Theißen and Saedler 2001). On the following, however, it suffices to confine the considerations to the simpler ABC model.

The evolutionary importance of homeosis

The heuristic value of homeotic mutants for understanding the development (ontogeny) of animals and plants is unquestionable. For example, homeotic mutants have been of great value for gaining a better understanding of how floral organs adopt their specific identity, as outlined above. Whether homeotic transitions represent a reasonable mode of evolution (phylogeny) remained a highly controversial issue, however. According to the still predominant scientific hypothesis, the 'Synthetic Theory' (or 'Modern Synthesis') of evolutionary biology, evolution always proceeds in a gradualistic manner, and hence drastic morphological changes such as homeotic transitions are of no evolutionary relevance (for a review, see Mauricio 2001, Theißen 2006). It is assumed that homeotic transformations require too much change to possibly confer selective advantage and thus always undermine the fitness of the affected organisms in such a serious way that there is strong selection against them (reviewed by Theißen 2006).

Despite all its merits, however, the Synthetic Theory, which is largely based on population genetics, might not be sufficient to explain important aspects of evolution. For example, it may fall short of explaining innovations and constraints, and the evolution of body plans (Riedl 1977, Gilbert et al. 1996, Wagner 2000, Haag and True 2001, Wagner and Müller 2002, Wagner and Laubichler 2004). In parallel to this insight, several lines of evidence have in recent years increased the plausibility of major evolutionary transitions brought about by individual mutations. For example, the analysis of 'Quantitative Trait Loci' (QTL) revealed that novel morphological forms in evolution may result from changes in just a few genes of large effect. Instructive examples are provided by the domestication of maize (*Zea mays*. ssp. *mays*) from teosinte (*Zea mays* ssp. *parviglumis*), and by species differences in monkeyflowers (*Mimulus* species) pollinated by bumble-bees (*Mimulus lewisii*) or hummingbirds (*Mimulus cardinalis*) (reviewed by Mauricio 2001).

The evolutionary relevance of major loci of large effect is difficult to reconcile with the view of the Synthetic Theory that morphological changes are based on mutations at many gene loci, each of small effect. Other major shortcomings of the Synthetic Theory, e.g. in explaining evolutionary novelties and constraints, led to the reintegration of developmental biology into evolutionary biology, giving rise to 'evolutionary developmental biology' ('evo-devo'). According to the rationale of evo-devo novel morphological forms in evolution often result from changes in developmental control genes (for details, see Gould 1977, Gilbert et al. 1996, Theißen et al. 2000, Carroll 2001, Arthur 2002).

Due to their potential to bring about drastic yet coordinate changes in the adult phenotype by modifying development, homeotic genes are especially attractive study objects of evo-devo projects. Some homeotic phenotypes (e.g. actinomorphic rather than zygomorphic flowers, four-winged rather than two-winged insects), resemble differences in character states between major organismic lineages. There is a long debate going on as to whether the genes underlying such 'phylomimicking mutants' define loci that play an important role in character changes during macroevolution (Kellogg 2000, Haag and True 2001). It is clear that changes in the expression domains of floral homeotic genes in mutant or transgenic plants can bring about homeotic transformations of floral organs. For example, the expression of class C genes in the whorls of the perianth leads to a transformation of sepals into carpelloid organs and of petals into stamenoid organs (Bradley et al. 1993). Similarly, the ectopic expression of class B genes in the 1st and 4th floral whorls of *Arabidopsis* leads to a transformation of sepals into petaloid organs and of carpels into stamenoid organs (Krizek and Meyerowitz 1996). Now there is evidence that such changes do not only underlay transgenic and mutant plants of negligible evolutionary potential, but also natural morphological diversity generated during macroevolution, and hence are suitable models for evolutionary processes. Tulips (*Tulipa gesneriana*) and other lily-like plants (Liliaceae), for example, have flowers displaying organ identities quite similar to the ones of higher eudicots, but first whorl organs are typically petaloid like second whorl organs rather than sepaloid. This suggests that a homeotic transition in the first floral whorl from sepaloid to petaloid organ identity, or vice versa, occurred during the evolution of flowering plants. Petaloid organ identity requires the function of class B floral homeotic genes (Fig. 2E). It did not come as a big surprise, therefore, that when putative class B genes were investigated in tulip, they were found to be expressed not only in the petaloid tepals of the second floral whorl, but also in the organs of similar identity in the first whorl (Kanno et al. 2003). Similar examples are provided by many flowers of the basal eudicot family Ranunculaceae, which have distinctly different petaloid organs in the first two whorls. Expression studies suggested that petaloidy of 1st whorl organs is due to a shift of class B gene expression towards the 1st floral whorl (Kramer et al. 2003).

These findings support the view that shifts in the boundaries of floral homeotic gene expression that brought about floral homeotic changes contributed to the diversity of floral architecture. They add to a growing stream of reasoning fuelled by evolutionary and cladistic analyses of morphological characters, all indicating that homeosis played a significant role in plant evolution (Sattler 1988; Iltis 2000; Kellogg 2000; Baum and Donoghue 2002; Rudall and Bateman 2002, 2003; Rutishauser and Moline 2005; for a recent review see Theißen 2006). Ronse De Craene (2003), for example, outlined several lines of evidence that the petals of the Rosaceae (comprising well-known cultivated plants such as rose, strawberry, apple, almond, apricot and peach) were derived from stamens.

An especially intriguing candidate case is provided by the enigmatic *Lacandonia schismatica*, which has 'inside-out flowers' with stamens in the centre of the flowers surrounded by carpels (Ambrose et al. 2006). This is arguably one of the most dramatic deviations from the typical floral ground plan known (usually, stamens surround the carpels in the centre of the flower), suggesting that a homeotic change affecting the reproductive organs occurred relatively recently during evolution in the lineage that led to *Lacandonia*. One should note, however, that the interpretation of the reproductive units of *Lacandonia* is still controversial; what appears to be flowers has alternatively been viewed as pseudanthia (Rudall 2003), so that the evolutionary developmental biology of *Lacandonia* needs further clarification. If the reproductive structures of *Lacandonia* are indeed true flowers, however, their origin very likely involved a considerable change in the ABC system of floral organ identity specification.

Models of how evolutionary variation of the ABC system of floral organ identity specification could explain floral diversification during evolution have been provided e.g. by Bowman (1997), Albert et al. (1998), Theißen et al. (2000) and Kramer et al. (2003). Consequences of homeotic transitions in evolution for our understanding of organ homology have been discussed by Theißen (2005).

Theoretically, homeotic changes could occur in a gradual mode during evolution (Sattler 1988). However, given that full conversions in organ identity usually take place in a mutant individual just by the mutation of a single homeotic gene, a saltational mode of character change appears more plausible, at least from a genetic point of view (Theißen 2006). This, however, would be difficult to reconcile with the Synthetic Theory, maintaining that all kinds of evolution are gradual and based on changes in allele frequency at many loci. And it would have a quite dramatic consequence: homeotic mutants should represent important steps during macroevolutionary transitions.

It's the ecology, stupid! – Floral homeotic mutants in the wild

Investigations following the evo-devo rationale and using the toolkit of developmental genetics have provided detailed information about as to how floral homeotic mutants can be generated in the first place. To establish novel structures in evolution, however, it does not suffice to generate a morphological feature by mutation in an individual organism; rather, the mutant allele must also go to fixation in a population in the wild. Unfortunately, evo-devo tells us little about the performance of homeotic mutants in natural environments – a crucial aspect for the evolutionary relevance of homeotic mutants (Theißen 2000). Non-gradual modes of evolution may not be generally accepted unless a sufficient fitness of respective mutants has been documented in natural habitats. To clarify that point the population dynamics of homeotic mutants has to be studied in extensive field work (Theißen 2000, 2006, Bateman and DiMichele 2002, Vergara-Silva 2003, Dietrich 2003).

Quite a large number of floral homeotic variants have been described in the literature (see e.g. Masters 1869, Darwin 1876, Murbeck 1918, Gottschalk 1971, Meyerowitz et al. 1989, Ronse De Crane 2003), but often heritability of the deviant phenotype – a requirement for any evolutionary relevance – remained unknown. And clearly, not even every mutant will do. Most of the 'classical' floral homeotic mutants, e.g. the ones that gave rise to the ABC model, have a dramatically reduced fitness, as is obvious already under laboratory conditions. The most extreme case is represented by class C loss-of-function mutants such as *agamous* of *A. thaliana*, which completely lack reproductive organs (Fig. 1C). Since *Arabidopsis* is not capable to reproduce vegetatively, such a mutant would soon become extinct in the wild. Class B null mutants (Fig. 1B), lacking stamens, are at least completely male sterile, but even class A mutants (Fig. 1A), although they have more rather than less reproductive organs in their flowers, may be hampered in their reproductive fitness.

A better way to identify floral homeotic mutants with evolutionary potential might be to watch out for such plants that appear in populations in the wild, thus revealing at least some minimum kind of competitiveness under natural growth conditions. Very few candidates that meet this criterium have been described, however. One is *bicalyx*, a recessive variety of *Clarkia concinna* (Onagraceae) in which the usually pink and showy trilobed petals are transformed into sepaloid organs due to a mutation at a single locus (Ford and Gottlieb 1992). The *bicalyx* variety occurs only in a small population north of San Francisco (Point Reyes, California, USA) accompanied by a majority (70%–80%) of wild type plants. Although the observation time of the population was just four years, it was reported to be stable, most probably due to the predominantly selfing mode of propagation (Ford and Gottlieb 1992).

Another case is a peloric variety of *Linaria vulgaris* (common toad-flax) that has actinomorphic rather than zygomorphic flowers and exists on a small island near Stockholm/Sweden (Cubas et al. 1999). Like the homeotic *Clarkia* also the *Linaria* is mutated in a single, recessive locus. However, while the *bicalyx* gene has not been molecularly characterized so far, it turned out that the *Linaria* variety is affected in a *CYCLOIDEA*-like gene, but by epimutation (methylation of DNA that leads to transcriptionally silencing) rather than change in the DNA sequence (Cubas et al. 1999). Both the *Clarkia* and *Linaria* varieties have a very limited range of distribution, and their fitness and competitiveness in the field has not been tested yet, but is questionable – the *Linaria* epimutant, for example, may only propagate vegetatively (Theißen 2000). The peloric variety of *Linaria* has been described for more than 200 years already, but whether its long-lasting existence on the island near Stockholm is due to true persistence of an ancestral mutant, or due to frequent reappearance after fast dieing out, has not been determined so far (Theißen 2000). Taking together, the evolutionary potential of the wild floral homeotic varieties reported so far is doubtful (Theißen 2006).

A floral homeotic variety of shepherd's purse

In contrast to the geographically very limited cases outlined above, a floral homeotic variety of shepherd's purse (*Capsella bursa-pastoris*) has been described for almost 200 years from different locations throughout Europe, and has been documented to exist in populations of remarkable size for a considerable number of years. This homeotic variety was termed '*dekandrisch*' ('decandric') (Opiz 1821), referring to the fact that its flowers have ten (greek: 'deka') rather than the usual six stamens of the wild-type (compare Fig. 2B, D with Fig. 2A, C). This is so because the four petals are transformed into stamens, hence the flowers of the decandric variety lack petals (Fig. 2B, D). Opiz (1821) considered the decandric variety a new species and named it '*Capsella apetala*'. Almost simultaneously decandric *C. bursa-pastoris* plants were found in Vienna/Austria (Trattinnick 1821) and in Braunschweig/Germany (Wiegmann 1823). Opiz (1821), Schlechtendahl (1823) and De Candolle (1827, cited after Dahlgren 1919) propagated *C. bursa-pastoris* plants via seeds and observed that the decandric phenotype is heritable.

Because decandric flowers exhibit additional stamens replacing petals, Murbeck (1918) termed the phenomenon '*Staminale Pseudapetalie*' ('Stamenoid pseudo-apetaly'). He analysed plants of different provenience (Sweden, Germany, Prague/Czech Republic and South Dakota/USA) and found flowers with petals transformed into almost normal stamens which, at least in some Swedish individuals, produced pollen. However, Murbeck (1918) also identified quite a number of individuals with flowers displaying changes other than ideal homeotic transformations, including the development of rudimentary organs or stamens that deviate from their normal form and do not produce pollen, lack of organs, and incomplete transformations from petals to stamens. Stamenoid pseudo-apetaly was also reported for many other flowering plants, such as the monocots meadow saffron (*Colchicum*), tulip (*Tulipa*), hyacinth (*Hyacinthus*), daffodil (*Narcissus*), *Iris, Crocus* and different orchids as well as many eudicots ranging from basal taxa such as *Ranunculus* and *Papaver* to a diverse range of higher eudicots (Murbeck 1918, Ronse De Craene 2003). Murbeck (1918) already considered the frequent occurrence of stamenoid pseudo-apetaly as of evolutionary relevance, assumed to reflect the origin of the perianth by conversion of stamens into petals. The interpretation of floral homeotic mutations as 'atavistic' changes that reproduce ancestral character states is highly controversial, however (e.g. Meyerowitz et al. 1989).

Dahlgren (1919) crossed various wild-types with decandric *C. bursa-pastoris* plants and found that in the F_1 generation the organs of the second floral whorl showed characteristics intermediate between petals and stamens. In the F_2 generation an approximately 1:2:1 segregation of individuals with stamenoid, intermediate and wild-type (petal) organs, respectively, in the second floral whorl appeared, suggesting that stamenoidy of 2nd whorl organs is co-dominant (or incompletely penetrant) to petaloidy and inherited by a single locus (or two or more closely linked loci). However, since a clear phenotypic distinction between intermediate and stamenoid second whorl organs was not always possible, Dahlgren (1919) counted them together and therefore reported a 1:3 segregation of wild-type and '*apetala*' plants.

In his seminal book on the importance of gene mutations for the evolution of plants Gottschalk (1971) dedicated a whole paragraph to the decandric variety of *Capsella*. Because of its sometimes massive occurrence in the wild he argued that the mutant variety must have a selective advantage compared to the wild type (a questionable argument, however, see below and Theißen 2006).

Besides brief mentioning in the literature (Meyerowitz et al. 1989) the decandric *C. bursa-pastoris* fell into oblivion for quite a while, until it was re-discovered in 1991 by Reichert (1998) on field paths in vine yards in Gau-Odernheim (Rheinhessen/Germany). Although not referring to the previous reports his description of the phenotype resembles those by Opiz (1821), Murbeck (1918) and Dahlgren (1919) in great detail. Reichert (1998) observed the population for several years and found the number and distribution of decandric plants to be quite stable, even though they are growing mixed with and embedded in populations of wild-type plants. The author noticed the peculiarity of a homeotic variant growing in the wild in a stable population of many plants rather than just appearing as a single mutant individual.

Studying *Spe*

As has been outlined above the decandric variety of *Capsella bursa-pastoris* is not the only floral homeotic mutant that has been found in the wild. Here we argue, however, that it has unprecedented features that make it an especially suitable system to study the evolutionary potential of floral homeotic mutants.

The relatively frequent and long-lasting presence in the wild guarantees that the fitness of the homeotic variety is at least not drastically hampered compared to that of the wild-type. Moreover, what appears important from an experimental point of view, *Capsella* is quite closely related to the major model plant *Arabidopsis*; both lineages may have diverged from one another about 10–14 million years ago (Koch and Kiefer 2005). This means that in contrast to many other floral homeotic mutants in the wild, the floral homeotic variety of *C. bursa-pastoris* can be rigorously studied, from the molecular genetic basis of the phenotype to its consequences on the ecology in the field, due to the fact that suitable experimental tools are available. Respective studies are underway and may help to answer the question as to whether non-gradualistic changes at the phenotypic level, such as homeotic transformations, have the potential to contribute to macroevolution (Theißen 2006).

We have identified additional decandric populations of *C. bursa-pastoris* throughout Europe (P.N. and B.N., unpublished data). Current work in our laboratories focuses on the populations from Gau-Odernheim (Reichert 1998) and an additional population found in the area of the Desenberg near Warburg (Westphalia/Germany). A careful analysis of the phenotype and mode of inheritance corroborated many of the findings reported in the literature (Opiz 1821, Trattinnick 1821, Murbeck 1918, Dahlgren 1919, Gottschalk 1971, Reichert 1998), suggesting – but by no means conclusively demonstrating – that the same gene(s) or even allele(s) are involved. Transformation of petals into stamens was complete or almost so in most of the mutant plants being investigated from both Gau-Odernheim (Fig. 2B, D) and Warburg. The pollen produced by the extra stamens proved to be viable and functional in fertilization (P.N., unpublished data).

Segregation of the mutant phenotype in the F_2 generation of crosses between mutant and wild-type plants varies, depending on the crosses being analysed, between stamenoid vs. petaloid 2nd whorl organs in a ratio of about 3:1, and stamenoid vs. intermediate vs. wild type second whorl organs in a ratio of about 1:2:1 (P.N., unpublished data). This suggests that the mutant phenotype of the homeotic populations in Gau-Odernheim and Warburg is caused by (a) (co-)dominant mutant allele(s) at a single locus, like for the varieties investigated before (Dahlgren 1919). Tests for allelism of the mutant loci in the Gau-Oderheim and Warburg populations are underway. Due to its phenotype and dominant mode of inheritance we have termed the locus/loci mutated in the decandric *C. bursa-pastoris* varieties "*Stamenoid petals*" (*Spe*).

Shull (1914) described a mutant with altered fruit shape in *Capsella bursa-pastoris* segregating in a Mendelian ratio of 1:15, suggesting that recessive alleles of two unlinked genes are responsible for the mutant phenotype. Shull (1914) concluded that capsule form in *C. bursa-pastoris* is determined by duplicate genes. In retrospect his observation can be explained with *Capsella bursa-pastoris* being a tetraploid plant. Our and the previous findings on the 3:1 (or 1:2.1) segregation of the *Spe* phenotype are not in conflict with these observations, considering that *Spe* is (co-) dominant rather than recessive, and that *C. bursa-pastoris*, despite being tetraploid, switched to a disomic mode of inheritance. We assume that the mutation conferring the *Spe* phenotype is just in one of the two genomes of *C. bursa-pastoris*.

According to the ABC model development of stamen identity requires class C floral homeotic gene activity, suggesting that in the *Spe* varieties class C gene expression is ectopically shifted towards the 2nd floral whorl, and class A gene activity is suppressed there (Fig. 2F). Ectopic class C gene function might be provided by an orthologue of the canonical class C gene of *Arabidopsis thaliana*, *AG*, or by any one of its closely related paralogues, *SHP1*, *SHP2* and *STK*, which share high sequence similarities with *AG* (Becker and Theißen 2003). Except *STK*, all these *AG*-type genes produce stamenoid organs in the 2nd whorl upon ectopic expression in *A. thaliana* (Favaro et al. 2003, and literature cited therein). In certain genetic backgrounds, e.g. *apetala2 agamous*, the *SHP*/*STK* genes are sufficient and required for carpel formation (Pinyopich et al. 2003). Due to the tetraploidy of the *C. bursa-pastoris* genome each of these loci might well be represented by two different (yet highly similar and related) genes in shepherd's purse, resulting in eight hot candidate genes for providing ectopic class C gene activity in the *Spe* variety. To test the modified ABC model for *Spe* (Fig. 2F), mRNA expression patterns of orthologues of class A, B and C floral homeotic genes in *Spe* and wild-type flowers of *C. bursa-pastoris* are currently being determined.

Scenarios not involving the ectopic expression of an *AG*-like gene cannot be excluded, but appear unlikely at the moment. This does not necessarily mean, however, that an *AG*-like gene is the *Spe* locus itself. In principle, ectopic expression of a class C gene could be brought about by a change in a *cis*-regulatory element of an *AG*-like gene itself, but also by a *trans*-acting factor functioning 'upstream' of the *AG*-like gene (and not necessarily directly binding to the *AG*-like gene itself). As we have learned recently, such an upstream-factor is not necessarily a protein, but could also be a regulatory RNA. For example, among the negative regulators of *AG* in the 1st and 2nd floral whorl is the non-MADS class A protein AP2, whose translation is inhibited by the microRNA miR172 in whorls 3 and 4 (reviewed by Steimer et al. 2004). It is thus conceivable that extension of the expression domain of miR172 from whorls 3 and 4 towards whorl 2 leads to an inhibition of AP2 synthesis and hence to an ectopic expression of *AG* in whorl 2.

Remarkably, the many screens for flower developmental genes in *A. thaliana* did so far not come up with a *Spe*-like mutant phenotype, at least to the best of our knowledge. Single-mutant flowers with fully stamenoid second whorl organs have typically also carpelloid first whorl organs (see, for example, Fig. 1A), suggesting differences in the regulation of floral organ identity between *Capsella* and *Arabidopsis* that deserve further studies.

Nevertheless, that *cis*- as well as *trans*-regulatory changes leading to ectopic expression of class C gene activity can bring about a *Spe* phenotype is even demonstrable in *A. thaliana*. Transformation of petals into stamens was realized in transgenic plants that express the class C gene *AG* under control of the promoter of the class B gene *AP3*, driving gene expression in the 3rd floral whorl – where *AG* is expressed anyway -, but also ectopically in the 2nd whorl (Fig. 3A, Jack et al. 1997). Even though an illegitimate recombination between a class C coding region and a class B gene regulatory region appears an extremely unlikely scenario for the origin of the *Spe* mutant locus in *C. bursa-pastoris*, the transgenic scenario nicely illustrates the feasibility of the modified ABC model depicted in Fig. 2F. It thus appears not unlikely that e.g. a mutation in a class C gene in *C. bursa-pastoris* destroyed a *cis*-regulatory element that is required to keep expression of the gene out of the 2nd floral whorl, especially since such a mutation could easily be dominant or co-dominant. An alternative scenario would be the addition of a 2nd whorl-specific enhancer to an *AG*-type gene, either by illegitimate recombination (as already mentioned above), or mutation. Even though this scenario appears less likely than the destruction of a negative regulatory element, the existence of 2nd whorl-specific regulatory sequences e.g. in the class B gene *AP3* (Hill et al. 1998) makes the respective event at least conceivable.

In contrast, loss-of-function mutations in trans-regulators of *AG* are more likely recessive rather than dominant. A *Spe* phenocopy in *A. thaliana* is even only known from a double mutant affected in two loci, *RABBIT EARS* (*RBE*) and *ROXY1* (Xing et al. 2005). Since a *rbe roxy1* double recessive mutant phenotype is unlikely to be established in natural populations except under circumstances of strong positive selection, we currently consider *Spe* being an *AG*-type gene a much more likely scenario, even though one certainly cannot exclude that floral gene regulation in *Capsella* is different from that in *Arabidopsis*.

Cloning of the *Spe* locus itself is, therefore, another important task for the future. We are currently applying a combined candidate and map based cloning approach which is facili-

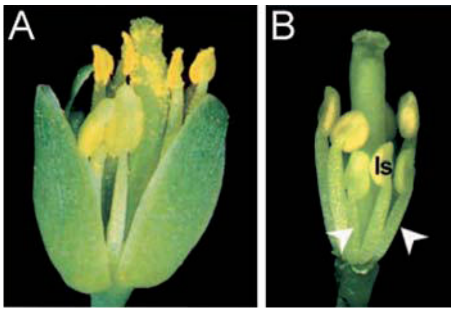

Fig. 3. Flowers of *Arabidopsis thaliana* with a *Spe* phenotype. **A** Flower of a transgenic plant in which the class C floral homeotic gene *AGAMOUS* is expressed under the control of the promoter of the class B gene *APETALA3* (AP3::*AG*), leading to the ectopic expression of the class C gene in the second floral whorl and hence the transformation of petals into stamens. **B** Flower of a *roxy1–3 rbe-2* double mutant; sepals were removed; white arrowheads point to extra stamens that develop in the second whorl. **A** is from Jack et al. (1997) with kind permission from Blackwell Publishing, Oxford, UK; **B** is from Xing et al. (2005) with kind permission from The Company of Biologists Ltd., Cambridge, UK. Abbreviation: *ls* lateral stamen

tated by the close relationship between *Capsella* and *Arabidopsis*. Comparative analysis revealed that the genome organization is still largely conserved between *Arabidopsis* and *Capsella* (Acarkan et al. 2000, Koch and Kiefer 2005). The genome sequence of *A. thaliana* has already been determined (Arabidopsis Genome Initiative, 2000), and sequencing of the *A. lyrata* and *C. rubella* genomes has been initiated (Joint Genome Institute, United States Department of Energy). All these findings and developments provide knowledge and tools facilitating gene mapping and cloning in *Capsella*.

Candidate genes for *Spe* currently being considered are all the *AG*-type genes mentioned above (orthologues of *AG*, *SHP1*, *SHP2*, *STK*), but also some *trans*-acting regulators of *AG* that show stamenoid petals upon mutation, such as *APETALA2* (*AP2*) (Drews et al. 1991, Deyholos und Sieburth 2000, Bomblies et al. 1999), *LEUNIG* (*LUG*, Liu und Meyerowitz 1995, Liu et al. 2000, Conner and Liu 2000), *SEUSS* (*SEU*, Franks et al. 2002), *CURLY LEAF* (*CLF*, Goodrich et al. 1997) and the already mentioned *RBE* and *ROXY1*. These putatively *trans*-acting candidates are considered with lower priority, however, because mutant alleles are usually recessive and mutant plants often display considerable pleiotropic effects beyond stamenoidy of petals, sometimes even outside of the flower. For example, Fray et al. (1997) described a double mutant of rape seed (*Brassica napus*) possibly affected in a *CLF* orthologue; its flowers very much resemble the *Spe* phenotype, but its vegetative leaves show a strong curly leaf phenotype known well from the original *clf* mutant of *Arabidopsis* (Goodrich et al. 1997). Restriction of the mutant effect to floral organs is characteristic for the *Spe* phenotype, however (Fig. 2B, D).

The origin and performance of *Spe* plants outside of the greenhouse is investigated both in ordered field plots in Botanical Gardens in Jena and Halle (Saale), and in their 'natural' environment in Gau-Odernheim and Warburg (which are typically man-made or disturbed habitats such as vineyards, however). Isozyme studies (Hurka et al. 1989, Hurka and Düring 1994) and AFLP-fingerprinting are being used to determine whether all *Spe* plants are monophyletic, even when growing at different locations, or whether the different *Spe* populations originated independently in the different habitats from wild-type plants.

Although *C. bursa-pastoris* is predominantly self-pollinating, even low rates of outcrossing could help to avoid inbreeding depression and hence might be of considerable importance for plant fitness and evolutionary potential. In case of the also predominantly selfing *A. thaliana* flower visits by potential pollinators such as solitary bees, dipterans and thrips have been observed in the field (Mitchell-Olds 2001, Hoffmann et al. 2003). In principle, the *Spe* variety could attract less, more, or the same number of floral visitors; moreover, the spectrum of floral visitors could be changed, for example because *Spe* flowers produce more pollen (preferred by beetles) than wild-type flowers, while the latter might be more attractive to bees and flies due to the presence of petals. To find out, we designed field plots in the Botanical Gardens of Jena (Fig. 4A) and Halle (Saale). Magnifying glasses were used to inspect individual flowers to identify small insects, such as thrips. Larger insects were caught by net or only counted by visual inspection, especially solitary bees (Figs. 4B, C), because many of them are endangered species. Especially species from the genus *Andrena* (Westrich 1990) were detected on both *Spe* and wild-type flowers of *C. bursa-pastoris* (J.Z., unpublished preliminary data). Two of these species are oligolectic, which means they are specialized to plant species belonging to the *Brassicaceae* family. So far we did not observe a dramatic change in the spectrum or number of floral visitors comparing *Spe* with wild-type plants (J.Z., unpublished preliminary data). Moreover, the numbers of fruits and seeds produced under our experimental conditions appears to be very similar for wild-type and *Spe* plants (J.Z., unpublished preliminary data). We thus did not find any evidence so far that the *Spe*

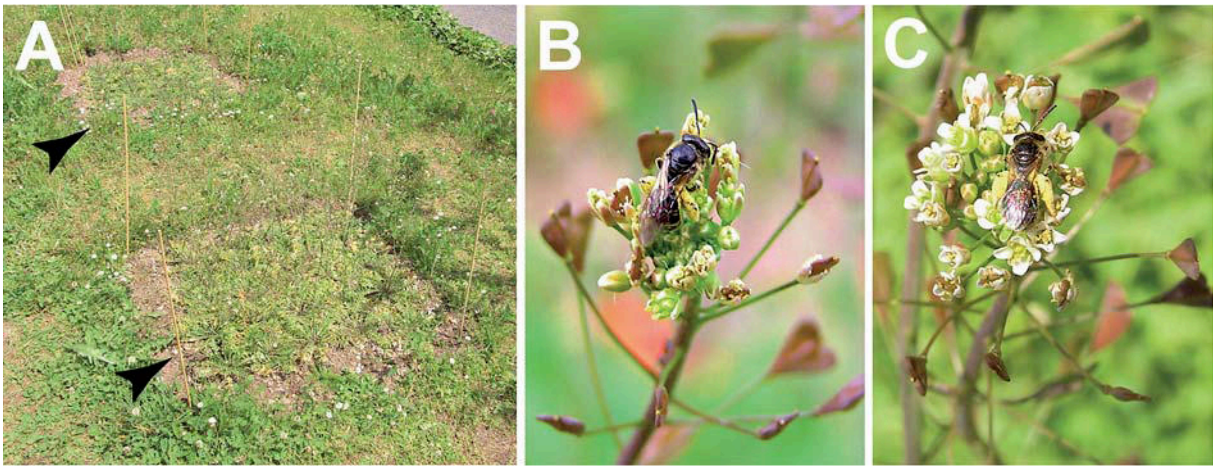

Fig. 4. Outdoor investigations of *Capsella bursa-pastoris* in the Botanical Garden of Jena. **A** Field plots (5 × 5 plants) of the *Spe* mutant (upper arrow) and the wild-type (lower arrow). **B, C** Solitary bees visiting the inflorescences of a *Spe* variety **(B)** and a wild-type plant **(C)**, respectively

variety is handicapped in its reproductive fitness, which is in line with its persistence in wild habitats for many years. Also because *Capsella bursa-pastoris* is mainly selfing rather than outcrossing these findings do not come as a big surprise.

Loss of perianth organs and increase in pollen production (e.g. by increasing stamen number) is a typical syndrome during evolution of wind-pollination, so represents the *Spe* variety a first step towards the evolution of wind-pollination within *Capsella*? This may sound absurd, but the consequences of the *Spe* mutation on outcrossing mediated by both floral visitors and wind will be determined in the botanical garden of Osnabrück/Germany. Moreover, the development of *Spe* populations in their natural habitats will be monitored as long as possible. Taken together, these endeavours will help us to learn more about the evolutionary potential of the *Spe* variety, and, hopefully, of floral homeotic mutants in general.

Summary and outlook

Comprising just three species *Capsella* is only a very small genus. However, with shepherd's purse it includes one of the most widely distributed flowering plants on our planet, and shows a number of interesting phenomena of parallel and convergent evolution affecting reproductive traits. In addition, with the *Spe* variety *C. bursa-pastoris* shows a very rare yet for the evolution of life on earth potentially very important phenomenon, namely the occurrence of a homeotic variety in quite stable populations in the wild. There is considerable circumstantial evidence that homeotic changes played a considerable role during the evolution of flowers, but the relevance of homeotic transformations during the origin of morphological novelties has remained a very controversial topic. Thus the *Spe* variety may help us to better understand one of the arguably most enigmatic aspects of life on earth, i.e. the origin of complex evolutionary novelties. Specifically, a detailed study of the *Spe* variety may not only tell us more about the developmental genetic mechanisms that generate novel form in the first place – something we know about quite a lot already -, but also whether and how drastic morphological variants are established in natural populations. Reconciling population genetics and macroevolution appears to be a considerable challenge for the future, and *Capsella* could be of great help here. Once *Spe* has been molecularly cloned, for example, it could be tested whether the locus is under purifying or positive selection. Another important question of future research will be to find out as to whether epimutations (e.g. methylation of DNA) serve as transitional steps in a 'trial phase' of mutant phenotypes during the establishment of morphological novelties (Theißen 2000).

So *Capsella* might be only a small genus, but it has great potential in evolutionary biology. Choosing it as a favourite model system for his investigations on the biogeography and phylogeny of plants about three decades ago was certainly a wise decision of Herbert Hurka. However, with the experimental tools currently becoming available it could well be that the best time of *Capsella* in evolutionary biology is yet to come.

This article is dedicated to Prof. Herbert Hurka on occasion of his 65th birthday, to honor his longstanding contributions to research on *Capsella*. The authors are grateful to Klaus Mummenhoff and Marcus Koch for their invitation to contribute to this volume. Many thanks to John Bowman for a number of valuable suggestions that helped to improve the manuscript. Many thanks also to Matthias Hoffmann (Halle Botanical Garden) and Conny Bartholmes, Steffen Hameister und Frank Buschermöhle (from our own laboratories) for discussions in the frame of our project on the *Capsella* variety *Spe*. Work on *Spe* in the authors' laboratories is supported by grants TH 417/4-1 and NE 314/7-1 from the Deutsche Forschungsgemeinschaft (DFG).

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4 Manuscript II

<u>P. Nutt</u>[1], Janine Ziermann[1] and G. Theißen (submitted 7th of May 2008 to *The Plant Cell*) Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural homeotic floral variant of *Capsella bursa-pastoris.*

([1] These authors contributed equally to this work)

Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural floral homeotic variant of *Capsella bursa-pastoris*

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ABSTRACT

We report the combined genetic and molecular analysis of a floral homeotic variant that occurs in natural populations in the wild. We demonstrate that the organs in the 2nd floral whorl of *Capsella bursa-pastoris* (shepherd's purse) variant "*Stamenoid petals*" (*Spe*) develop into functional stamens rather than petals, while the identity of all other floral organs and the timing of organ formation is the same as in wild-type flowers. Genetic analyses revealed that *Spe* is a co-dominant allele of a single locus, and that two populations in Germany with a *Spe* phenotype are affected at the same locus. In an F_2 mapping population, the *Spe* phenotype co-segregates with the *CbpAGa* locus, but not with any of the seven other members of the clade of *AGAMOUS*-like genes in the genome of the tetraploid *C. bursa-pastori*s. *In situ* hybridization analyses showed that the *AGAMOUS* co-orthologues of *C. bursa-pastoris* are ectopically expressed in primordia of 2nd whorl organs of *Spe* flowers, thus explaining why these organs develop into stamens rather than petals. As the only major sequence difference between wild-type and mutant alleles, we identified a deletion of 22 base pairs in a highly conserved region of the 2nd intron of *CbpAGa*. Our findings strongly suggest that *Spe* is a mutant allele of *CbpAGa* in which a previously unrecognized negative *cis*-regulatory element of *AGAMOUS* genes, which keeps class C homeotic gene expression out of the 2nd floral whorl, has been deleted.

INTRODUCTION

Homeotic mutants show a type of variation in which "something has been changed into the likeness of something else" (Lewis, 1994). They are frequent in plants, affecting both vegetative and reproductive organs (Sattler, 1988; Meyerowitz et al., 1989). Floral homeotic mutants have more or less normal floral organs in places where organs of another type are typically found. The model plant *Arabidopsis thaliana* (thale cress) has flowers that consist of four different types of organs, i.e. sepals, petals, stamens and carpels, which are arranged in four whorls. In *A. thaliana* homeotic mutants have been categorized into three classes termed A, B and C (Coen and Meyerowitz, 1991). Ideal class A mutants have carpels instead of sepals in the 1st floral whorl, and stamens rather than petals in the 2nd whorl. Class B mutants contain sepals rather than petals in the 2nd and carpels rather than stamens in the 3rd whorl. And class C mutants have flowers in which reproductive organs (stamens and carpels) are replaced by perianth organs (petals and sepals, respectively), and in which the determinacy of floral growth is lost, resulting in an increased number of floral organs (Meyerowitz et al., 1989).

The defined classes of floral homeotic mutants have been explained by the ABC model (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Krizek and Fletcher, 2005). It proposes three different floral homeotic functions to explain how the different floral organs adopt their identities during development. Corresponding to the mutant classes these functions are termed A, B and C, with A specifying sepals in the 1st floral whorl, A+B petals in the 2nd, B+C stamens in the 3rd and C carpels in the 4th whorl. To account for class A and C mutant phenotypes it was proposed that the A function and the C function work antagonistically, so that the A function is expressed throughout the flower in a C loss-of-function mutant, and *vice versa* (Coen and Meyerowitz, 1991).

In *Arabidopsis* the class A genes are represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the class B genes by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the (single) class C gene by *AGAMOUS* (*AG*). All these genes encode putative transcription factors (reviewed in Krizek and Fletcher, 2005). Except for *AP2* all ABC genes are MADS-box genes encoding MIKC-type MADS-domain transcription factors (reviewed in Becker and Theißen, 2003).

The molecular mechanism by which class A, class B and class C proteins interact involves another class (E) of MIKC-type transcription factors termed SEPALLATA (or AGL2-like) proteins (Pelaz et al., 2000; Honma and Goto, 2001; Ditta et al., 2004). According to the floral quartet model (Theißen and Saedler, 2001) the proteins encoded by the class ABC genes bind to the class E proteins in a combinatorial way to constitute multimeric regulatory complexes that specifically recognize *cis*-regulatory elements ('CArG-boxes') of their target genes. Hence it goes without saying that the study of floral homeotic mutants has told us a great deal about the development of floral organ identity.

In contrast, whether floral homeotic mutants and genes play a role in evolution is a highly contentious topic. On the one hand, there is considerable circumstantial evidence that they do; on the other hand this is difficult to reconcile with the predominant evolutionary theory which rejects any drastic change of the phenotype as reasonable mode of evolution due to its assumed negative impact on the fitness of the affected organism. Anyway, structural diversity of flowers, such as differences between the flowers of *Arabidopsis*, columbine, tulip and orchids, has been explained to quite some extend by modifications of the ABC system of floral organ identity specification, especially by changes in the spatiotemporal expression domains of the ABC genes which lead to homeosis (Kanno et al., 2003; Kramer et al., 2003; Mondragón-Palomino and Theißen, 2008). Beyond that, there is further evidence provided by evolutionary analyses of morphological characters of both extant and extinct plants that homeosis played a significant role in plant evolution (Sattler, 1988; Kellogg, 2000; Baum and Donoghue, 2002; Rudall and Bateman, 2002, 2003; Ronse De Craene, 2003; Theißen, 2006).

Like all other mutants, homeotic mutants originate as rare individuals in populations of wild-type organisms. To establish new evolutionary lineages homeotic mutants thus need to survive many years under conditions of natural selection until the mutant homeotic locus went to fixation and possibly modifying mutations have fine-tuned and optimized the new 'body design'. How does this work? Unfortunately, the mechanisms which explain how homeotic mutants originate tell us little about the performance of homeotic mutants in natural ecosystems (Theißen, 2000). Thus to determine the evolutionary potential of floral homeotic mutants, their population dynamics has to be studied in extensive field work (Theißen, 2000, 2006; Bateman and DiMichele, 2002).

There is little doubt that most floral homeotic mutants have their fitness so strongly reduced that their long-term survival in nature is almost certainly hampered (Nutt et al., 2006). Thus probably only a small fraction of homeotic mutants will have sufficient fitness under natural growth conditions to establish new evolutionary lineages. A reasonable method to identify such mutants might be to look for populations of floral homeotic variants in the wild. However, up to now only few of such populations have been described in the literature. One case is *bicalyx*, a variant of *Clarkia concinna* (Onagraceae) in which the petals are transformed into sepaloid organs (Ford and Gottlieb, 1992); another example is a peloric version of common toad-flax (*Linaria vulgaris*) with actinomorphic rather than zygomorphic flowers (Cubas et al., 1999). Both are mutated at a single, recessive locus. It turned out that the toadflax variant is affected in a *CYCLOIDEA*-like gene by methylation of DNA (epimutation), but for the *bicalyx* variant no molecular data has been reported so far (Cubas et al., 1999).

Both the *Clarkia* and *Linaria* variants described have a very limited range of distribution, and their fitness in the wild is probably significantly lower than that of the wild-types, so that their evolutionary potential is questionable at best (Theißen, 2000). To develop a more comprehensive understanding of the evolutionary importance of floral homeotic mutants - and homeotic variants in general – model systems are required that can be studied at different levels of biological complexity, ranging from the molecular mechanisms that bring about the variant phenotype to the ecology in the field (Nutt et al., 2006; Hintz et al., 2006; Bateman and Rudall, 2006). Studying the molecular developmental genetics of a system efficiently requires tools that are only available for so called 'model organisms'. Populations of natural homeotic variants have not been reported for the predominant flowering plant model system *A. thaliana* so far. Therefore, we started a research program on *Stamenoid petals* (*Spe*), a remarkable variant of *Capsella bursa-pastoris* (shepherd's purse), which is a close relative of *A. thaliana*. In the flowers of the *Spe* variant the four petals appear to be transformed into stamen-like organs, while all other floral organs seem to be unaffected (Nutt et al., 2006; Hintz et al., 2006).

As outlined in detail elsewhere, the *Spe* variant has been described at different places in Europe for almost 200 years now. One quite large and stable population on field paths in vine yards in Gau-Odernheim (Rheinhessen/Germany) has been monitored for about 20 years (Nutt et al., 2006). The long-lasting existence of the *Spe* variant in the wild indicates that its fitness cannot be dramatically different from that of the wild-type with which it co-exists.

Like *A. thaliana*, *C. bursa-pastoris* is self-compatible and easy to cultivate and propagate. Albeit longer than that of *A. thaliana*, the life cycle of *C. bursa-pastoris* allows the analysis of three to four generations per year. And even though *C. bursa-pastoris* is a tetraploid plant, it shows disomic inheritance (Hurka et al., 1989), which makes crossing experiments easier to interpret. Because of the close relationship between the genus *Capsella* and *A. thaliana* numerous experimental tools can be adapted quite easily to study the *Spe* variant. We have shown already that *C. bursa-pastoris* is amenable to genetic transformation by the ‘floral dip’ method (Bartholmes et al., 2008), which facilitates the analysis of gene function. Moreover, the order, orientation and sequence of genes are very similar in *Arabidopsis* and *Capsella*, with more than 90% sequence identity within exons (Boivin et al., 2004; Koch and Kieffer, 2005). This allows the identification of genes within *Capsella* with the help of the *Arabidopsis* genome. We are thus confident that the *Spe* variant of *C. bursa-pastoris* represents an excellent system to study all aspects of the developmental and evolutionary biology of a floral homeotic mutant.

Here we provide a detailed report about flower development in the *Spe* variant compared to that of the wild-type, involving two populations. We investigated the mode of inheritance employing crosses between wild-type and *Spe* plants, and the molecular mechanism by which the *Spe* mutant phenotype is brought about. Based on the ABC model we hypothesize that in the *Spe* mutants ectopic expression of a class C gene, or a closely related gene, is extended from the 3rd and 4th whorl towards the 2nd whorl and thereby suppressing class A genes in this whorl (Nutt et al., 2006). Most obvious candidate genes for this ectopic expression are an orthologue of the *Arabidopsis* class C gene *AGAMOUS* or one of its closely related paralogues *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*) (Becker and Theißen, 2003). For all of these genes, except *STK*, it has been shown that their ectopic expression in *A. thaliana* leads to the formation of stamen-like organs in the 2nd whorl (Pinyopich et al., 2003). To test our hypothesis we investigated mRNA expression patterns of the orthologues of class A, B and C floral homeotic genes, and their closely related paralogues, in wild-type and *Spe* flowers of *C. bursa-pastoris*. Finally, we report identification of a candidate mutation in an allele of a putative class C floral homeotic gene that co-segregates with the mutant phenotype.

Our findings represent a valuable basis for a rigorous and comprehensive analysis of the evolutionary importance of homeosis in an experimentally tractable model system.

RESULTS

Structure, development and function of *Spe* inflorescences, flowers and floral organs

Wild-type plants of *Capsella bursa-pastoris* develop flowers that are very similar to those of *Arabidopsis thaliana*, including four sepals in the 1st floral whorl, four white petals in the 2nd, six stamens in the 3rd and two fused carpels in the 4th whorl (Figure 1A, C). Individual flowers of *C. bursa-pastoris* are rather inconspicuous, but at anthesis many flowers of an inflorescence are grouped together in a corymboid structure that may serve as a recognition unit for floral visitors (Figure 1E).

In the flowers of both *Spe* variants investigated in this study, 1947-*Spe* and 1948-*Spe*, the organs of the 2nd flower whorl are transformed into stamen-like organs, thus raising the overall number of stamens or stamen-like organs per flower from six (in the wild-type) to ten, while no petals develop anymore (Figure 1B, D). The floral organs of all other whorls appear unchanged. Consequently, the inflorescences of *Spe* plants (Figure 1F) appear yellow-greenish and thus may be less attractive for visually oriented visitors than wild-type inflorescences (Figure 1E).

Since *Spe* flowers differ from wild-type flowers of *C. bursa-pastoris* mainly (if not exclusively) in the homeotic transformation of the organs in the 2nd floral whorl, these stamen-like organs were analysed in more detail. Wild-type stamens of *C. bursa-pastoris* typically consist of a long filament topped by the anther, which contains two thecae with two pollen sacs separated by the connective (Figure 1G). 3rd whorl stamens of *Spe* variants are indistinguishable from those of wild-type plants. The ectopic stamens in the 2nd whorl have the same general structure as wild-type stamens, but their filaments are shorter and their anthers are more variable in size, ranging from slightly smaller to slightly bigger than those of typical 3rd whorl organs (Figure 1H, I). Scanning Electron Microscopy (SEM) revealed that the epidermis structure of the anther of 2nd whorl stamens is very similar to that of *Spe* 3rd whorl stamens as well as stamens of wild-type plants (Figure 1J-L). In all cases cell margins are lobed and the moderately bulged surface is ridged by cuticular waxes as it was shown previously for *Arabidopsis thaliana* anthers (Smyth et al. 1990). Also, filament surfaces showed identically long rectangular cell shapes in the two *Spe* variants as well as in wild-type stamens. In both *Spe* lines the 2nd whorl stamens contain approximately half the amount or even less pollen than 3rd whorl stamens of *Spe* variants or wild-type plants.

Detailed analysis also revealed different peculiarities in the two *Spe* lines. In the variant 1947-*Spe* some anthers, in addition to having short filaments, are longer and wider than the usual ones. In these anthers the yellow colour appears brighter and the pollen sacs contain even less pollen compared to other *Spe* stamens (Figure 1M, N). Very few of the anthers show incompletely developed and empty pollen sacs and form a short petaloid blade at their tip (Figure 1O). The surface structure of those white coloured areas consists of smaller, isodiametric and dome shaped cells similar to the epidermis of wild-type petals (Figure 1P, Q), indicating some remnants of petaloidy. In the variant *Spe*-1948 the anthers do not show petaloid residuals, but they are sometimes smaller than 3rd whorl stamens, and they contain less pollen (Figure 1I). Very few of these anthers seem to be less properly developed, with occasionally only one pollen sac per theca. Here, laceration sometimes fails so that pollen remains kept inside of the anther (Figure 1R). However, the epidermis surface of these smaller anthers was indistinguishable from those of 3rd whorl stamens in SEM analysis (Figure 1K, L).

In summary, with few exceptions the 2nd whorl organs of *Spe* plants resemble perfect stamens.

To determine whether 2nd whorl stamens of *Spe* plants do not only resemble stamens morphologically, but also functionally, we tested whether these organs produce viable pollen and whether this pollen is fertile. Pollen of variants 1947-*Spe* (Figure 1T) and 1948-*Spe* (data not shown) were treated as described by Alexander (1969) and showed the same pink to purple staining as pollen of wild-type plants (Figure 1S), indicating that the ectopic stamens of *Spe* plants produce viable pollen like the ordinary stamens of wild-type plants do.

The fertility of the pollen of ectopic stamens was tested by pollination of emasculated flowers of wild-type and *Spe* plants with the pollen of 2nd whorl stamens of both *Spe* lines (Supplemental Table 1 online). Except for two experiments, where fertilization failed, this yielded about the same number of seeds per fruit (12.8-24.5) as in positive control experiments in which pollen of 3rd whorl *Spe* stamens was used, or in which flowers of *Spe* and wild-type plants were simply left untreated for selfing as positive controls for fertilisation success (Supplemental Table 1). Seed set in all these experiments was an order of magnitude higher compared to negative control experiments in which stamens in flowers of both *Spe* lines were completely removed to provide an estimate for pollen-contamination during the preparation process (Supplemental Table 1). These data demonstrate that ectopic 2nd whorl stamens produce functional pollen like 3rd whorl stamens do.

Taken together, our data show that the *Spe* lines represent natural homeotic floral variants in which 2nd whorl organs develop into functional stamens.

We next investigated whether *Spe* flowers show not only homeotic, but also heterochronic changes. As a prerequisite for respective studies we established an overview about the stages of wild-type flower development in *C. bursa-pastoris* based on landmark events (Supplemental Table 2 online) similar to the more detailed one provided by Smyth et al. (1990) for the close relative *A. thaliana*. It turned out that the development of *C. bursa-pastoris* wild-type flowers (Figure 2A, C) very much resembles that of *A. thaliana* flowers. The only major deviation is that petals develop more slowly when compared to carpels, so that the carpel tube is closed at the tip and starts to develop stigmatic papillae before petal tips reach level with the tips of lateral stamens (Figure 2C).

When the landmark events of flower development of the *Spe* variants 1947-*Spe* and 1948-*Spe* were compared to that of the wild-type, no significant deviations were observed (Figure 2B). There is also no change in the number or arrangement of floral primordia produced by the inflorescence meristem, or of floral organ primordia generated by the floral meristem. Also in *Spe* flowers organ development follows exactly the defined chronology observed for wild-type flowers (Figure 2A-D). Most importantly, the transformed 2nd whorl organs of both *Spe* variants show the same retardation of organ outgrowth as the petal organs of wild-type plants do (Figures 2D). This indicates that even though the 2nd whorl organs of *Spe* flowers are homeotically transformed, their development reveals no heterochronic changes, but follows the timing of wild-type 2nd whorl organs. Since in *Spe* flowers the development of the 2nd whorl stamens is delayed compared to the 3rd whorl stamens, the completion of outgrowth and the subsequent opening of the thecae and the pollen release occur later in these organs. Also late organ development seems not to be changed compared to normal petal development in terms of chronology. Carpel and fruit development shows no obvious difference between wild-type and *Spe* lines. Furthermore, the alternating 2nd whorl organ position and the assignment of abaxial and adaxial sides of the transformed organs are not affected (Figure 2).

A co-dominant allele of a single genetic locus is responsible for the *Spe* phenotype

To determine the transmission of the *Spe* phenotype to progeny 1947-*Spe* and 1948-*Spe* plants were selfed for six or four generations, respectively. Like the parental plants all offspring developed inflorescences in which all flowers showed a full *Spe* phenotype as described (e.g., Figure 1B), while 1947-wt plants never developed *Spe* flowers when selfed for several generations. Our observations indicate that the *Spe* phenotype is stably inherited with perfect penetrance and full expressivity over several generations, suggesting that it is based on a true mutation rather than an epigenetic change. Moreover, the fact that no wild-type plants occurred after selfing of *Spe* plants indicates that the parental plants were homozygous for all loci relevant for the *Spe* phenotype.

To identify the mode of phenotype expression both *Spe* lines were crossed with wild-type plants in reciprocal directions. The F_1 generations of all these crossings showed intermediate floral phenotypes, i.e. 2nd whorl organs with features of petaloidy and stamenoidy, while the organs in all other floral whorls remained wild-typic (Figure 1U-W). The organs of the 2nd whorl differed in their degree of stamenoidy, ranging from weakly stamenoid organs mainly composed of petal blades (Figure 1V, right) to more strongly stamenoid organs composed of chimaeric antheroid/petaloid blades (Figure 1V, left). These more or less severely affected organs occurred apparently in a random way within single flowers. The surface of the chimeric organs shows isodiametric and dome-shaped cells characteristic for wild-type petals in the blade area as well as longitudinal ridged cells in the stalk epidermis which identify them as filament-like organs (data not shown). Even in case of the more severely affected stamenoid organs, however, pollen was not produced. These observations indicate that in 2nd whorl organs petaloidy and stamenoidy, respectively, are determined by wild-type and mutant alleles of *Spe* in a co-dominant way.

To determine the number of genetic loci controlling stamenoidy of organs in the 2nd floral whorl analyses of F_2 populations of wild-type x mutant plants and reciprocal testcrosses were performed (Table 1). Three phenotypic classes were observed: wild-type flowers, intermediate flowers as those observed in F_1 generations and full mutant flowers with 2nd whorl organs (almost) completely transformed into stamens, as known from 1947-*Spe* and 1948-*Spe* plants.

When the numbers of intermediate and full *Spe* phenotypes are taken together, the segregation patterns resulted in a ratio of one wild-type (genotype +/+) to about three mutant plants (*Spe*/+; *Spe*/*Spe*), thus fitting to a model of dominant inheritance of a single mutant locus (Table 1). This "1:3" model is well supported by p-values close to 1. If all three phenotypic classes are counted individually, the numbers result in a segregation ratio of one wild-type (+/+) to about two intermediate (*Spe*/+) and one *Spe* phenotype (*Spe*/*Spe*) with p-values ranging from 0.26 to 0.94. This "1:2:1" model reflects a co-dominant mode of inheritance (Table 1).

We took a closer look at the expressivity of the intermediate and the full *Spe* phenotypes in the cross where the lowest p-value was found (1a in Table 1). Among the F_2 generation, 31 individuals developed intermediate as well as *Spe* organs in one flower, which were initially counted as intermediate phenotypes and thus skewed segregation ratios. To test them for homozygosity at the *Spe* locus, they were selfed and the F_3 generations were analysed for segregation (Supplemental Table 3 online). Among the 31 F_3 generations seven were found, in which no segregation of the *Spe* phenotype was observed, indicating that their parental plants were homozygous. Taking these seven plants into account in an additional analysis, a new segregation ratio with a p-value of 0.95 was obtained (1b in Table 1). These data demonstrate a reduced expressivity of 1947-*Spe*/1947-*Spe* homozygous mutant alleles when crossed into a 1947-wt background.

In contrast to these observations the analysis of crosses involving 1948-*Spe* showed a higher expressivity indicated by a better approximation to 1.0 in p-values. Additionally, differences in the severeness of intermediate organ transformation was usually visible in intermediate F_2 plants of the two different *Spe* line crossings, with 1948-*Spe* yielding stronger stamenoidy of 2nd whorl organs than 1947-*Spe* (Figures 1U, W).

The same genetic locus is responsible for the *Spe* phenotype in two geographically distant populations

1947-*Spe* and 1948-*Spe* represent plants from two populations in Germany which are about 200 km apart. To determine whether the same (or closely linked) loci are mutated in the two populations, we developed a specific crossing experiment that takes the co-dominant nature of the mutant alleles causing the *Spe* phenotype into account.

First, the two homozygous lines 1947-*Spe* and 1948-*Spe* were crossed in both directions (Table 2.1), resulting in F_1 generations that showed a perfect *Spe* phenotype. A plant of each F_1 generation was selfed and the offspring (F_2) was analysed. Concerning the genetic basis of the *Spe* phenotype in 1947-*Spe* and 1948-*Spe* we considered two extreme possibilities. If in both populations the same locus (or more than one but genetically closely linked loci) cause organ transformation in the 2nd floral whorl we expected only genotypes that generate a *Spe* phenotype (Table 2.2). If, however, the *Spe* phenotype is caused by different, genetically unlinked loci a segregation ratio of 1:15 typical for two-factor crosses, with 1 wild-type to 15 mutant (intermediate and *Spe*) phenotypes, was expected. In both offspring generations only *Spe* mutant phenotypes were found (Table 2.2), suggesting that the *Spe* loci in 1947-*Spe* and 1948-*Spe* are allelic (or closely linked).

To corroborate our conclusions the"mixed heterozygous" plants generated in the F_1 generations were backcrossed with homozygous 1947-wt plants in both directions, resulting in several offspring generations to be analysed for segregation (Table 2.2). Again two extreme scenarios were considered. In case of 1947-*Spe* and 1948-*Spe* being affected at the same locus an intermediate phenotype was expected for all offspring; in case of different, unlinked loci the typical two-factor backcross segregation pattern was expected, with genotypes 1 (+/+; +/+) to 1 (*Spe*/+; +/+) to 1(+/+; *Spe*/+) to 1(*Spe*/+; *Spe*+) that result in a relation of 1 wild-type to 3 mutant phenotypes (Table 2.2). Except in one case (see below) in the offspring generations either only mutant phenotypes (selfed offspring) or only intermediate phenotypes (backcross with the wild-type) were found (Table 2.2), strongly supporting our hypothesis that in 1947-*Spe* and 1948-*Spe* the same loci are mutated. However, the intermediate plants showed a very broad spectrum of phenotypes, ranging from hardly distinguishable from wild-types to almost perfect *Spe*-like, with typical intermediate forms being more frequent than extreme ones. Generally, the offspring of reciprocal cross B had the distribution of phenotypes shifted towards less severe petal transformation compared to cross A. Many of the analysed offspring plants showed only few intermediate organs while the majority of flowers was wild-type. In one exceptional case (Table 2.2, cross no. 6) we even found a considerable number of plants without traces of mutant phenotype and hence classified them as "wild-type". We assume, however, that this represents just an extreme case of low penetrance of the mutant phenotype. Hence, our data suggests that expression of the *Spe* mutant phenotype depends on mutant background.

Expression patterns of floral organ identity genes in *Spe* compared to wild-type flowers

To determine whether the expression of floral homeotic genes is changed in *Spe* compared to wild-type flowers, detailed studies employing *in situ* hybridization were done. To generate specific hybridization probes we isolated cDNAs of the genes of interest with a focus on members of the *AGAMOUS* clade of genes. In *C. bursa-pastoris* we found two distinct sequences for every gene from *A. thaliana*, which very likely reflects the tetraploidy of the

C. bursa-pastoris genome. Since *C. bursa-pastoris* has disomic rather than tetrasomic inheritance we treated the sequence variants as different loci rather than alleles here, distinguished by suffixes "a" and "b".

Complete coding sequences where isolated from *CbpAGa* and *CbpAGb* (putative co-orthologues of *AGAMOUS*), *CbpSHP1a* and *CbpSHP1b* (putative co-orthologues of *SHATTERPOOF1*), *CbpSHP2a* and *CbpSHP2b* (putative co-orthologues of *SHATTERPOOF2*), and *CbpSTKb* (putative co-orthologue of *SEEDSTICK*). Of *CbpSTKa* only a partial sequence containing 5´-UTR, MADS-box and part of the I-region was isolated from cDNA. To test whether additional *AGAMOUS* clade members are present in the *C. bursa pastoris* genome we performed genomic DNA gel blot ('Southern') hybridization experiments using genomic sequences (including introns) spanning most part of the I region to the start of the C-terminal region as probes. Except for *CbpSHP1* probes, where four bands were observed in case of one restriction enzyme being used (Supplemental Figure 1C online, lanes 1, 7), just one or two bands were detected with both wild-type and *Spe* genomic DNA (Supplemental Figure 1). In some cases a two band pattern in a lane can be traced back to cutting sites in the digested genomic DNA (Supplemental Figure 1). These results corroborate that at least three of the four *AG*-clade members from *A. thaliana* have two co-orthologues in the *C. bursa-pastoris* genome, which could also be true for *SHP1*, in which case the four bands would represent allelic polymorphisms rather than additional genetic loci. However, the presence of additional copies of *CbpSHP1* genes beyond *CbpSHP1a* and *b* cannot be excluded at the moment.

Correct classification of the gene pairs as co-orthologues of their *A. thaliana* counterparts was verified through phylogenetic reconstructions with these sequences in comparison to other angiosperm *AG* clade members (sequence alignment and phylogenetic tree in the Supplemental Figures 2 and 3 online, respectively).

For *CbpAP3a/b, CbpPI*a/b and *CpbAP1a/b*, orthologues of the respective floral homeotic genes from *A. thaliana*, as well as for one *CbpH4* (*Histone 4*) gene only partial sequences were isolated. Due to high sequence similarity, co-orthologous sequences are expected to cross-hybridize. On the following, therefore, we do not distinguish between sequence variants "a" and "b".

Standard *in situ* hybridization protocols were adapted to the flowers of *C. bursa-pastoris* employing probes for a *CbpH4* gene, which is mainly transcribed in replicating cells (Brandstädter et al., 1994; Groot et al., 2005). Wild-type and *Spe* floral tissues showed the same characteristic punctuate expression pattern in which only replicating cells are strongly stained (Figure 3A, B), suggesting that cell division activity is not drastically altered in *Spe* compared to wild-type flowers.

CbpAP3 and *CbpPI* showed very similar expression patterns in *C. bursa-pastoris* as the class B floral homeotic genes *AP3* and *PI*, their orthologues in *A. thaliana*. Differences between wild-type and *Spe* flowers were not observed (Figure 3C-J). Starting already at developmental stage 2 and stage 3, floral buds express *CbpAP3* in a circular zone between centre and margin of the bud, where organs of the 2nd and 3rd flower whorl will arise (Figure 3C, D). In the following stages, the signal was mainly restricted to developing organs of those whorls (Figure 3E-H). From about stage 8 onwards, when 2nd whorl organ primordia are still small but filaments and thecae of 3rd whorl stamens already developed, weak expression of *CbpAP3* was also detected in developing carpel tissue (Figure 3F, H). Expression of *CbpAP3* was detected in the upper parts of petals and in anthers of 2nd and 3rd whorl stamens until stage 12 (Figure 3G, H). At early developmental stages of wild-type and mutant plants expression of *CbpPI* showed the same pattern as *CbpAP3*, in that the signal was detected in regions where 2nd and 3rd whorl organs will arise (Figure 3I, J).

In both wild-type and *Spe* inflorescences, strongest *CbpAP1* expression was found in the floral primordia of stage 1 and 2 (Figures 3K-M). At stage 3 expression of *CbpAP1* was still detectable in the sepal primordia and the adjacent cells leading to the central dome of the floral bud in wild-type plants (Figure 3N). But at stage 6, when first primordia of 2nd whorl became visible, *CbpAP1* expression was not detectable anymore (Figures 3O, P). Differences in *CbpAP1* expression between wild-type and *Spe* flowers were not apparent.

The expression of *CbpAG* in wild-type floral buds (Figure 4A) was not easy to detect and to distinguish from background staining probably because of a low expression level. Earliest *CbpAG* expression was detected in stage 3 and 4 buds in the centre of the floral meristem of both wild-type and *Spe* plants (Figure 4A-C). At stage 6, when stamen and carpel primordia in the 3rd and 4th floral whorl are well developed and the tiny primordia of 2nd whorl organs arise, *CbpAG* expression was detected in the organs of the two inner whorls of both wild-type and *Spe* flowers (Figure 4D-F). Only in *Spe* flowers, however, *CbpAG* shows also a staining signal in the small organ primorida of the 2nd whorl (Figure 4E). Even at later developmental stages *CbpAG e*xpression remains to be restricted to the organs of the 3rd and 4th whorl in wild-type flowers, whereas in *Spe* flowers also in developing 2nd whorl organs *CbpAG* expression was detected (Figure 4G-I). In flowers after stage 9 expression is limited to rapidly developing tissues like ovules and stigmatic papillae in carpel and anthers of the 3rd whorl stamens and, in *Spe*, also in the 2nd whorl stamens (Figure 4J-L). In flowers of this age we also detected *CbpAG* expression throughout the nectaries at the base of the 3rd whorl stamens in both the wild-type and in *Spe* (Figure 4J, and data not shown).

CbpSTK expression at stage 4 was detected in young stamen and carpel primordia (Figure 5A-C). At stage 9 a signal was not detectable any more in wild-type flowers (Figure 5D), but in *Spe* flowers mild expression in developing stamens and carpels was still visible (Figure 5E). In addition to the expression in 3rd and 4th whorl organs, ectopic expression of *CbpSTK* was also found in the stamens developing in the 2nd whorl, where expression appears stronger than in the 3rd whorl (Figure 5E). However, strongest expression of *CbpSTK* was detected at late stages in developing ovules of both wild-type and *Spe* plants as it was predictable from the knowledge about *Arabidopsis* flower development (Figures 5G-I).

Neither expression of *CbpSHP1*, nor of *CbpSHP2* was detected in any of the early developmental stages of floral buds in wild-type (data not shown) and *Spe* plants (Figures 5J-L). Only at later stages, when ovules developed in the carpel, we could detect weak expression signals at stage 8 floral bud of the *Spe* variant in the marginal cell layers of the placental zones of the ovule (Figure 5M). This demonstrated that expression of *CbpSHP1* and *CbpSHP2* could be detected under our conditions, but since such a late expression is very likely irrelevant for the *Spe* phenomenon (organ identity in the 2nd whorl), we did not study it in a comparative way. Nevertheless, we examined fruits after fertilisation and found *CbpSHP1* and *CbpSHP2* expression in the valve margins as well as in the endothelium of ovules, exemplarily shown in Figure 5N-Q. In summary, *CbpSHP1* and *CbpSHP2* expression patterns are similar to those in developing *Arabidopsis* flowers (Flanagan et al., 1996).

***CbpAGa*, but none of the seven other *AG*-like genes, co-segregates with the *Spe* mutant phenotype**

Our previous results suggested that a mutation in either an orthologue of *AGAMOUS* or *SEEDSTICK*, or a negative regulator of the transcription of these genes in the 2nd floral whorl is responsible for the *Spe* phenotype. We thus checked the co-segregation of mutant- and wild-type-specific alleles of all *AG*-like genes in an F_2 population obtained by a cross between 1947-wt and 1947-*Spe*. To identify sequence polymorphisms distinguishing alleles from the wild-type and *Spe* parent we amplified and sequenced about 5 – 7.5 kbp of genomic sequence from each of the parents for loci *CbpAGa*, *CbpAGb*, *CbpSHP1a*, *CbpSHP1b*, *CbpSHP2a*, *CbpSHP2b*, *CbpSTKa* and *CbpSTKb*. For each locus sequence comparisons identified 4-16 candidates for sequence polymorphisms distinguishing parental alleles. All except one were located in non-coding regions such as introns and regions upstream of the coding region. As marker for the co-segregation analysis at least one SNP (Single Nucleotide Polymorphism) per gene was verified by pyrosequencing genomic DNA of the parents and the F_1 generation.

Genotyping of 9-11 wild-type plants of the F_2 population by pyrosequencing revealed recombination between the candidate loci under investigation and the floral phenotype for all genes except one, *CbpAGa* (Table 3). This implies that *CbpAGb*, *CbpSHP1a*, *CbpSHP1b*, *CbpSHP2a*, *CbpSHP2b*, *CbpSTKa* and *CbpSTKb* cannot represent the *Spe* locus, leaving *CbpAGa* as the only remaining candidate. We thus checked for co-segregation between the informative SNP and the *Spe* phenotype in the whole F_2 population, comprising 196 plants (Supplemental Table 4 online). Segregation of both phenotype and SNP followed again a 1:3 ratio model supported by a p-value of 0.9. For 191 plants both genotype and phenotype data could be determined, revealing a perfect co-segregation between SNP character state and phenotype (Supplemental Table 4). This reveals that *Spe* and *CbpAGa* are genetically closely linked, or even identical, loci.

A detailed sequence comparison between the *CbpAGa* alleles from the parental plants revealed a total of 16 sequence polymorphisms, all upstream of the coding region and in the 1st and 2nd intron. 15 of the corresponding mutations are very unlikely the cause of the *Spe* phenotype. Most of these represent short microsatellites or polybase pair-stretches in little conserved regions, the others are SNPs representing autapomorphies in little conserved regions of the *CbpAGa* sequence of wild-type plants, as revealed by multiple sequence alignments (data not shown). The only remaining sequence difference is a combined putative deletion of 22 bp and a substitution of three bp in the 2nd intron (Figure 6). Considerable conservation of the corresponding region in *AG* orthologues throughout the Brassicaceae reveals that this indel almost certainly represents a deletion in the *CbpAGa* allele of the mutant (*Spe*) parent rather than an insertion in the allele from the wild-type parent (Figure 6). This characteristic deletion is absent in the *CbpAGa* allele of 1948-*Spe*, the other *Spe* line investigated here, as revealed by PCR analysis (see Supplemental Figure 4 online).

DISCUSSION

We are establishing the *Spe* variant of *C. bursa-pastoris*, a plant that is known from wild habitats for at least about 200 years, as a model system to investigate the evolutionary potential of floral homeotic mutants (see also Theißen, 2006; Nutt et al., 2006; Hintz et al., 2006). Within this framework, the goal of the studies described here was to clarify the phenotype and molecular developmental genetics of the *Spe* variant.

The *Spe* variant as a new model for a widespread phenomenon in flowering plants

A detailed morphological investigation involving plants from two populations revealed that the *Spe* lines represent a natural floral homeotic variant in which 2nd whorl organs develop into (almost) perfect stamens from both a morphological and functional point of view. Changes in other floral whorls, or any other pleiotropic effects on vegetative organs, were not observed. This phenotype is remarkable in that it displays a full homeotic conversion in organ identity in only one floral whorl, since in the typical floral homeotic mutants of the close relative *A. thaliana* always two adjacent whorls are affected (Coen and Meyerowitz, 1991).

The syndrome of petals transformed into stamens is known for many flowering plants, such as the monocots tulip (*Tulipa*), hyacinth (*Hyacinthus*), daffodil (*Narcissus*), meadow saffron (*Colchicum*), *Iris, Crocus* and different orchids, as well as for many eudicots, including *Ranunculus*, *Papaver* and a number of higher eudicots (Murbeck, 1918; Ronse De Craene, 2003). While our analysis strongly suggests that the *Spe* phenotype of *C. bursa-pastoris* is based on mutation of a single locus, it has not been observed so far in a single gene mutant of *A. thaliana*, despite the numerous mutagenesis experiments that have been carried out with this species. Except for transgenic plants a *Spe* phenotype in *A. thaliana* is only known from the *rbe roxy1* double recessive mutant affected in two loci, *RABBIT EARS* (*RBE*) and *ROXY1* (Xing et al., 2005). Therefore, with the molecular cloning of the *Spe* gene potential regulatory differences between *C. bursa-pastoris* and *A. thaliana* concerning the specification of organ identity in the 2nd floral whorl, may soon become apparent. Such differences may exist, for example, due to the tetraploidy of the *C. bursa-pastoris* genome. However, conservation of the site mutated in *CbpAGa*, the *Spe* candidate locus, suggests that similar mutations could be generated also in other Brassicaceae. It would be interesting to investigate, therefore, whether the *CbpAGa* mutation identified in the 2nd intron of *Spe* plants in a full-length genomic clone of the *AGAMOUS* locus transformed into wild-type *A. thaliana* generates a dominant *Spe* phenotype. If so, one could conclude that it's only by chance that such a mutation did not appear in *A. thaliana* yet.

In any case, the mutant variant of *C. bursa-pastoris* described here provides us with a new, experimentally tractable model system to investigate the *Spe* syndrome, a phenomenon that is widespread in flowering plants, but has been neglected by molecular plant research so far, possibly because of its absence in the model plant *A. thaliana*.

Spe **genetics - simple but revealing**

Our genetic analyses demonstrated that co-dominant inheritance of the *Spe* phenotype in both populations, 1947-*Spe* and 1948-*Spe*, is stable and caused by a change in DNA sequence (rather than an epimutation) at a single genetic locus, even though the involvement of two or more closely linked loci cannot be completely ruled out. The segregation ratios observed corroborated the long held but little tested view that *C. bursa-pastoris*, despite its tetraploid genome, has disomic rather than tetrasomic inheritance (Hurka et al., 1989).

Our analyses revealed that 1947-*Spe* and 1948-*Spe* are mutated at loci that are at least closely linked, with the by far most simple and likely hypothesis being that the *Spe* loci in plants from both populations are allelic.

Segregation patterns of F_2 populations that fit into 1:2:1 models demonstrated that in a heterozygous condition the *Spe* allele controls stamenoidy of the organs in the 2nd floral whorl in a co-dominant fashion. This mode of inheritance indicates that the *Spe* effect is gene (better: allele) dose dependent. Moreover, we hypothesize that a co-dominant allele is more likely to represent a gain-of-function mutation than a recessive allele. Remarkably, gain-of-function often results from the ectopic expression of a gene. Prominent examples involving genes encoding transcription factors include *Knotted1*, *Gnarley1* and *Rough Sheath1* from maize, and *Hooded* from barley (Vollbrecht et al., 1991; Foster et al., 1995; Schneeberger et al., 1995; Müller et al., 1995). We consider it quite likely, therefore, that the *Spe* phenotype is brought about by the ectopic expression of a developmental gene controlling stamen identity. An intriguing precedent case is provided by the *Ovulata* (also known as *Macho*) mutant of snapdragon (*Antirrhinum majus*), which results from the insertion of a transposon into the 2nd regulatory intron of the *AGAMOUS* orthologue *PLENA* (Bradley et al., 1993). This created a dominant gain-of-function allele of *PLENA* that is ectopically expressed in the outer two whorls of the flower, resulting in mutant flowers in which sepals are replaced by carpels, and petals by stamens (Bradley et al., 1993). Despite the fact that stamenoidy of 2nd whorl organs is accompanied by carpelloidy of 1st whorl organs, the similarity to the *Spe* system at the genetic level is striking. We therefore tested how far this similarity goes down to the molecular level.

Ectopic expression of two organ identity genes accompanies organ transformation in *Spe* flowers

According to the ABC model we hypothesize that a class C gene, or a close relative, is ectopically expressed in the 2nd floral whorl of *Spe* flowers, while the expression of class A genes is reduced, and that of all other organ identity genes is unchanged in all whorls.

To test our hypothesis we studied the expression of orthologues of floral organ identity genes of *A. thaliana* in *Spe* and wild-type flowers of *C. bursa-pastoris*. In addition to orthologues of *AGAMOUS* (class C), *APETALA1* (class A), and *APETALA3* and *PISTILLATA* (class B) we considered also *SEEDSTICK* and *SHATTERPOOF1* and *2*, since they show high sequence similarity to *AG*. Expression of class E (*SEPALLATA*) genes was not determined, since their function is essential for the development of all floral organ identities. Also the A-class gene *AP2* was not tested, because in *A. thaliana* it is expressed in all floral organs and regulated post-transcriptionally (Jofuku et al., 1994).

Expression of the putative class A floral homeotic gene *CbpAP1* was analysed in order to test whether the antagonistic regulation between class C and class A floral homeotic genes known from *A. thaliana* (Mandel et al., 1992, Gustafson-Brown et al., 1994) is also operating in *C. bursa-pastoris*. We detected *CbpAP1* only at early developmental stages, which is in contrast to the *AP1* expression in *A. thaliana*. Our observations did not provide any evidence for regulatory interactions between *CbpAP1* and *CbpAG*, including repression of *CbpAP1* by *CbpAG* activity. We hypothesize that in *C. bursa-pastoris* early expression of *CbpAP1* is predominantly responsible for conferring floral meristem identity, whereas a function as organ identity gene is less well developed or even absent, due to the early termination of expression.

Since the development of petals and stamens requires class B floral homeotic gene expression, differences in the expression of these genes between wild-type and *Spe* flowers were not expected. And indeed, expression patterns of the putative class B floral homeotic genes *CbpAP3* and *CbpPI* were found to be indistinguishable in wild-type and *Spe* flowers of

C. bursa-pastoris, and very similar to those of *AP3* and *PI*, respectively, in *A. thaliana* flowers (Jack et al., 1992; Goto and Meyerowitz, 1994).

The most obvious candidate genes for providing the ectopic class C gene activity are an orthologue of the canonical *Arabidopsis* class C gene *AGAMOUS*, or one of its closely related paralogues *SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*), which are all members of the clade of *AG*-like genes (Becker and Theissen, 2003). Our expression analysis of the genes of the *AG*-clade demonstrated that *CbpAG* and *CbpSTK*, orthologues of *AG* and *STK*, are ectopically expressed in the organs developing in the 2nd floral whorl of *Spe* flowers. Besides this ectopic expression *CbpAG* transcription signals in the inner two whorls of the flower were very similar to that in wild-type *C. bursa-pastoris* and *A. thaliana* flowers (Bowman et al., 1991; Drews et al., 1991). In contrast to that, the expression of *CbpSTK* in young stages of 3rd and 4th whorl organs of both wild-type and *Spe* flowers shown here has not been described for *Arabidopsis* flowers (Rounsley et al, 1995; Colombo et al., 1995). For orthologues of the *SHP* genes, *CbpSHP1* and *CbpSHP2*, no signals of expression in flower buds before developmental stage 8 were detected, and never in developing stamens, strongly suggesting that the *SHP* orthologues are not involved in the homeotic transformation in *Spe* flowers.

It is known that ectopic expression of *AG*, *SHP1* or *SHP2*, but not *STK*, is sufficient to transform 2nd whorl floral organs into stamens in the flowers of *A. thaliana* (Pinyopich et al., 2003). Remarkably, transformation of petals into stamens without other changes, was observed in transgenic plants that express *AG* under control of the promoter of the class B gene *AP3*, driving gene expression in the 3rd floral whorl – where *AG* is expressed anyway -, but also ectopically in the 2nd whorl (Jack et al., 1997). This demonstrates that ectopic expression of *AG* restricted to the 2nd floral whorl is, at least in *Arabidopsis*, sufficient to bring about a phenotype perfectly resembling *Spe*. Our findings suggest that a similar mechanism brings about the *Spe* phenotype.

Assuming that also in *C. bursa-pastoris* the *AG* gene, but not the *STK* gene confers stamenoidy, the question remains why both genes are ectopically expressed in *Spe* flowers. We have shown that the *Spe* phenotype is very likely caused by mutation at a single locus, and it is *a priori* unlikely that both genes are mutated in *Spe* plants. Our mapping data demonstrate that the *Spe* phenotype, and hence ectopic expression of *CbpAG*, is linked to the *CbpAGa* locus, but not to *CbpSTK*. Therefore, it appears plausible that *CbpAG* expression activates *CbpSTK*. This is in line with the fact that in *A. thaliana*, *AG* has the broader function in specifying stamen as well as carpel development, while the *STK* function is restricted to aspects of carpel (i.e., ovule) development (Pinyopich et al., 2003, Favaro et al. 2003). This means that in *C. bursa-pastoris*, *CbpSTK* being a target of *CbpAG* is quite conceivable.

Ito et al. (2007) have shown that in *A. thaliana* stamen development requires prolongued *AG* activity, as *AG* works there over different transcriptional cascades in different floral stages. Our finding that *CbpAG* is continuosly expressed during 2nd whorl stamen development in *Spe* flowers strengthens our hypothesis that the development of ectopic stamens is controlled by *CbpAG*.

Taken together, our findings strongly suggest that the *Spe* phenotype in *C. bursa-pastoris* is brought about by the ectopic expression of a *CbpAG* gene in the organs of the 2nd floral whorl, as summarised in a model in Figure 7, even though an involvement of *CbpSTK* cannot be excluded.

Molecular cloning of the *Spe* gene by a candidate gene approach

Concerning the ectopic expression of a *CbpAG* gene in the 2nd floral whorl, the *Spe* variants may be either mutated in one of the two *CbpAG* loci, or in a direct or indirect negative regulator of *CbpAG*. Our linkage analysis (Table 3) eliminates with one strike 7 out of 8 *AG*-like genes (one *CbpAG*, four *CbpSHP* and two *CbpSTK*) as candidate genes for the *Spe* locus. In contrast, the *CbpAGa* allele from mutant plants perfectly co-segregates with the mutant *Spe* allele in a mapping population involving 191 plants, indicating that the respective loci are genetically closely linked. We conclude that *Spe* and *CbpAGa* are located on the same chromosome in close vicinity with a distance probably below 1 cM. The simplest hypothesis would be that *Spe* is just a mutant allele of *CbpAGa* rather than a *trans*-acting regulator closely linked to *CbpAGa*. On the following, we provide cumulative evidence strongly supporting that view.

In *A. thaliana*, quite a number of negative *trans*-acting regulators of *AG* are known, such as *APETALA2* (*AP2*) (Jofuku et al., 1994), *LEUNIG* (*LUG*) (Liu und Meyerowitz, 1995), *SEUSS* (*SEU*) (Franks et al., 2002), *CURLY LEAF* (*CLF*) (Goodrich et al., 1997), *BELLRINGER* (BLR) (Bao et al., 2004), *EMBRYONIC FLOWER1* and *2* (*EMF1, 2*) (Calonje et al., 2008), *RABBIT EARS* (*RBE*) (Krizek et al., 2006), *STERILE APETALA* (*SAP*) (Byzova et al., 1999) and *ROXY1* (Xing et al. 2005). In contrast to *Spe* from *C. bursa-pastoris*, however, mutant alleles are usually recessive, and mutant plants often show incomplete transformation of petals into stamens (if any), and/or display considerable pleiotropic effects beyond stamenoidy of petals, sometimes even outside of the flower. For example, Fray et al. (1997) described a double mutant of *Brassica napus* (rape seed) possibly affected in an orthologue of *CLF*. Mutant flowers very much resemble the *Spe* phenotype, but vegetative leaves show a strong curly leave phenotype. In the *roxy1* and *rbe* single mutants of *A. thaliana* mainly organs of the 2nd floral whorl are affected, but complete changes in organ identity (homeotic transitions) are not observed (Xing et al. 2005; Krizek et al., 2006). In *BELLRINGER* mutants, carpelloid sepals rather than stamenoid petals are observed (Bao et al., 2004). We hypothesize, therefore, that the complete floral homeotic transitions seen in *Spe* are more likely due to a mutation in a floral homeotic gene rather than a *trans*-acting regulator. Moreover, we observed that none of the negative regulators of *AG* known so far is genetically closely linked with the *AG* locus. Since the order, orientation and sequence of genes are very similar in the genomes of *Arabidopsis* and *Capsella* (Boivin et al., 2004), it is likely that the same is true for orthologous genes in the genome of *C. bursa-pastoris* as well. In that case, *Spe* cannot be such a regulator of *CbpAG*, because we have shown that both loci are closely linked. It thus appears more likely that the *Spe* phenotype is caused by a mutation in a *cis*-regulatory element in the sequence of *CbpAGa* itself. Such a regulatory element would be required to keep expression of the gene out of the 2nd floral whorl, e.g. by binding a negative *trans*-acting factor.

It thus appears striking that the only major sequence difference distinguishing the *CbpAGa* allele of wild-type and *Spe* plants that we could identify affects a highly conserved region in the 2nd intron. The very long intron of *AG* genes (about 3.5 kb in *A. thaliana*) is well known for its regulatory function not only in *Antirrhinum* (*PLENA* gene, see above), but also in *A. thaliana* (see e.g. Sieburth and Meyerowitz, 1997; Busch et al., 1999; Deyholos and Sieburth, 2000, Hong et al., 2003). This intron contains numerous binding sites for *trans*-acting factors, comprising both activators and repressors of *AG* activity. The sequence polymorphism distinguishing *CbpAGa* from *Spe* and wild-type plants is located in the 5' region of the second intron, at about 30 % of relative intron length. In line with our findings, this part of the intron has previously been demonstrated to be responsible for stamen development in *A. thaliana*, whereas the 3' part was shown to be involved in regulating carpel and ovule development (Deyholos and Sieburth, 2000). We hypothesize that in case of the polymorphism discussed here a sequence motif responsible for negative regulation of *CbpAGa* in the 2nd floral whorl of *C. bursa-pastoris* is partially or fully deleted. This sequence motif may well bind one (or more) *trans*-acting factor, most likely a protein working as a transcription factor or transcriptional co-regulator.

Quite a number of *trans*-acting factors directly or indirectly binding to the 2nd intron of *AG* are known (reviewed in Liu and Karmakar, 2008). Positively acting regulators, such as *LEAFY* (*LFY*) and *WUSCHEL* (*WUS*), are not considered here further, because the *Spe* phenomenon is thought to be based on abolishment of binding of a negative regulator. Also for some, but not all, negative regulators acting via the 2nd intron binding sites are known (Bao et al, 2004, Nole-Wilson and Krizek 2000). *APETALA2* (*AP2*) and *AINTEGUMENTA* (*ANT*) are both members of the AP2/EREBP-gene family encoding transcription factors and are known as negative regulators of *AG* in *A. thaliana*, but their consensus binding site differs strongly from the polymorphic site identified here (Jofuku et al 1994; Krizek et al. 2000; Nole-Wilson and Krizek 2000)**.** *LEUNIG* (*LUG*) and *SEUSS* (*SEU*), encoding interacting members of different protein families, are two other negative regulators of *AG* (Franks et al. 2002). They do not bind directly to DNA, however, but are probably recruited to the DNA of the 2nd intron by binding to dimeric complexes of MADS-domain proteins AP1, AGL24, SEP3 or SHORT VEGETATIVE PHASE (SVP) (Gregis et al., 2006; Sridhar et al., 2006; Liu and Karmakar, 2008). MADS-domain proteins bind to CArG-boxes (consensus 5'-CC(A/T)$_6$GG-3'), but such DNA-sequence elements are not obvious in the *CbpAGa* polymorphic site considered here (Figure 6). The BELLRINGER (BLR) protein is also a supposed binding partner of LUG and/or SEU, but its known binding site in the *AG* 2nd intron of *A. thaliana* is located about 500 bp downstream of the polymorphic site and differs clearly in its sequence (Bao et al., 2004). *PETAL LOSS* (*PTL*) and *ROXY1* are also negative regulators of *AG* in *A. thaliana*, but are very likely not involved in recognising the polymorphic site as they work indirectly by posttranslational modification of other repressors (Brewer et al., 2004, Xing et al., 2005).

Currently the arguably most likely candidate for a factor binding to the sequence deleted in the putative *Spe* allele could be the orthologue of *RABBIT EARS* (*RBE*), because the function of *RBE* is restricted to the 2nd whorl of the flower and not much information is currently available that rules out its ability to bind to a putative binding motif located in the sequence polymorphism of *Spe*. *RBE* encodes a Zn-finger protein whose DNA-binding motif contains a AGT-core sequence (Krizek et al., 2006). Such a motif is apparent in the polymorphic site (Figure 6), and another one in close proximity, but the probability to find such a short motif by chance is already quite high in any random sequence of three nucleotides (1:64). Moreover, the *rbe* loss-of-function phenotype in *A. thaliana* is much weaker than the *Spe* phenotype, so in *CbpAGa* more than the binding site of just one factor has possibly been deleted.

Nevertheless, *Spe* could be a yet unknown gene that, just by chance, is closely linked to the *CbpAGa* locus, and not related to the ectopic *CbpAGa* expression. Taken together, however, all pieces of evidence fall so nicely into place that we are quite confident that with *CbpAGa* we have cloned the *Spe* locus already. We thus favour the by far most simple and arguably most likely hypothesis that *Spe* is a mutant allele of *CbpAGa* in which a previously unrecognized negative *cis*-regulatory element in the 2nd intron, which is involved in keeping class C homeotic gene expression out of the 2nd floral whorl, has been deleted.

If the sequence change in the 2nd intron of *CbpAGa* is the critical mutation of the *Spe* locus in 1947-*Spe*, we can already conclude that 1948-*Spe* contains a different allele that lacks the respective deletion (Supplemental Figure 4). This suggests that both alleles, and thus probably also both populations of *Spe* plants, originated independently from wild-type alleles/populations. Whether it is just by chance or due to some intrinsic instability that the same locus has been affected in both *Spe* populations remains to be seen until even more populations have been investigated.

Definitive evidence that *Spe* is an allele of *CbpAGa* requires further experimentation, e.g. transformation of the mutant genomic locus from a *Spe* plant, including all regulatory sequences upstream and downstream of the coding region, into a wild-type plant of *C. bursa-pastoris*. Transformation protocols for *C. bursa-pastoris* that may facilitate such work have recently been developed (Bartholmes et al., 2008). Since *Spe* is co-dominant, transformants should show a *Spe* or intermediate phenotype, thus demonstrating that *CbpAGa* is *Spe*. If different populations with a *Spe* phenotype show different mutations, but in the same locus, this would also corroborate the hypothesis that *Spe* is an allele of *CbpAGa.*

In any case, cloning of the *Spe* locus opens the door for investigations on the molecular evolution of a gene that may facilitate saltational evolution by homeosis.

METHODS

Plant material

Capsella bursa-pastoris (L.) Medik. selfed offspring was used from individuals of the wild-type line ‘wt 6’, and individuals from *Spe*-variant lines ‘*Spe* 9 and *Spe* 8. All these lines originated from population ‘1947’, located in Gau-Odernheim (Rheinhessen, Germany). Additional selfed offspring used was from line ‘*Spe* 2’, of the population ‘1948’, located near Warburg (Westfalen, Germany) (Nutt et al., 2006). The plant lines are here referred to as ′1947-*Spe*′, ′1947-wt′ and ′1948-*Spe*′, respectively (Bartholmes et al., 2008). *Capsella* population and plant line numbers refer to the Brassicaceae Germ Plasm Collection of the Department of Systematic Botany, University of Osnabrück, Germany.

To test for stable inheritance members of each plant line were arbitrarily chosen and continuously selfed for four or six generations, resulting in two complete inbred lines of 1947-*Spe* and one in each case of 1948-*Spe* and 1947-wt, respectively.

Genetic crosses

Plants serving as female parents were emasculated by removal of all organs except for the carpel a day before the floral buds would have been opened and after a day off for maturation of the stigma they were pollinated. Members of all F_2 generations were selfed after analysis and seeds stored at 4°C. Pedigree diagrams in Supplemental Figure 5 online show how genotypes for the genetic analysis were generated.

Microscopy

Flowers, floral organs and pollen staining were observed under a binocular microscope LEICA MZ-FLIII. *In situ* hybridizations have been documented with a LEICA DM 5500B microscope. Samples for Scanning Electron Microscopy (SEM) were fixed in FAEG Fixative (formaldehyde 3%, acetic acid 5%, ethanol 65%, glutaraldehyde 0.2%) containing 0.1% Tween 20 for 16-20 h at 4°C. The tissue was dehydrated through an ethanol series up to 100% alcohol followed by an alcohol-acetone series up to 100% acetone. After critical point drying with CO_2, samples were spottered with gold. Pictures were collected on a Phillips XL 30 ESEM scanning electron microscope.

Test for Pollen functionality

Viability of the pollen grains was tested with Alexander´s Stain (Alexander, 1969), containing malachite green which stains cellulose in pollen walls turquoise, and acid fuchsin which stains the pollen protoplasm purple. The stock stain solution was diluted 1:50 with 10% acidic acid for differentiation. Already opened anthers with the pollen mass presented were soaked with the diluted stain solution and immediately photographed under a binocular microscope. 2nd and 3rd whorl stamens both from varieties 1947-*Spe* and 1948-*Spe* (five flowers per plant; three plants per line) were analysed, as well as stamens from one 1947-wt plant (five flowers).

In fertility tests flowers from pollen recipients (“mother” plants) were emasculated by removing sepals and all stamens in the latest bud stages shortly before opening to avoid self-fertilisation in the crosses. Thereafter carpels of these plants were pollinated with pollen from 2nd whorl stamens of pollen donor (“father”) plants. The tested plant lines and control crossings are listed in Supplemental Table 1 online. This procedure was carried out daily over a time span of two to three weeks. As a measure for fertilisation success seed numbers per fruit were scored.

Molecular cloning of cDNA fragments

The cDNA from total RNA of *C. bursa-pastoris* inflorescences was generated with Oligo d(T)- containing primers according to standard protocols. The 3´ and 5`RACE (Rapid Amplification of c-DNA Ends) were performed using the 5´/3´RACE kit (Roche, Mannheim, Germany) according to the manufacturer´s instructions. Primers used for amplification will be provided upon request.

For phylogenetic analysis of the genes *CbpAG, CbpSHP1, CbpSHP2* and *CbpSTK* full length sequences including the UTRs were generated with primers derived from UTR sequences obtained in the 3´ and 5´ RACE-cloning (for primer sequences see Supplemental Table 5 online).

In general all PCR-fragments (including genomic fragments) were gel-purified and cloned into pGEM-T (Promega), pJET1 or pBluescript II SK(+) (both Fermentas) and afterwards sequenced with the respective vector primers.

In situ hybridization

Organ primordia of the 2nd and 3rd floral whorl and developmental stages have been checked for correct identification by inspection of cutting series through the respective flowers.

Influorescences were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.1% Tween 20 (16h at 4°C). Post fixation steps, dehydration by Histo-clear, embedding, sectioning and prehybridization was carried out according to the protocol of Zachgo (2002) except triethanolamine treatment was skipped. Samples were digested with 1 µg/ml Proteinase K in 2 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.0, for 30 min. Templates for *in vitro* transcription were generated by PCR and contain a T7 RNA polymerase binding site either at the 3´end (antisense probes) or the 5´end (sense probes). Either at the 5´end (anti-sense probes) or the 3´end (sense probes) a recognition site for the restriction enzyme *XbaI* or *XhoI* was introduced and digested after template generation by PCR (for primers see Supplemental Table 5 online). All template sequences lack the MADS domain to avoid cross hybridization and have the 3´UTR included (for an alignment of probes see Supplemental Figure 6 online, and for primers Supplemental Table 5). *In vitro* transcription, hydrolysis of probes to an approximate length of 150 bp, hybridization, post hybridization washes and immunological detection were also performed according to the protocol of Zachgo (2002). Usually 0,5µg - 1µg DIG- labelled probe/ 100µl hybridization solution was used. Hybridization was carried out at 50-52°C for 12-16 h and the final washing steps were done for 2 x 30 min in 0,3 SSPE at 52-53°C. The detection buffer contained 10% PVA and slides were incubated for 14-20 h.

Co-segregation analysis

To generate a segregating F_2 population *C. bursa-pastoris* 1947-wt and 1947-*Spe* (pollen donor) were crossed. Afterwards one plant of the resulting F_1 generation was selfed to obtain the F_2 population of which 191 (from a total of 196) plants analysed. For all F_1 and F_2 plants the phenotype was determined during flowering.

Isolation of genomic DNA from leaf material was done with the standard methods. Amplification of genomic DNA was done via three and two step standard PCR protocols. Putative promoter sequences were generated by genome walking following the protocol from CLONTECH Laboratories. Sequences of oligonucleotide primers used for amplification will be provided upon request.

Amplification of the short fragments from genomic DNA necessary for the subsequent genotyping was done via a standard PCR protocol. For pyrosequencing we followed the procedure published by Groth et al. (2006). Sequences of respective oligonucleotide primers used for fragment amplification and the following pyrosequencing reaction are listed in Supplemental Table 5 online. To genotype the complete F_2 population for the SNP in *CbpAGa* we ordered a biotinylated forward primer to pyrosequence directly without ligation step (Supplemental Table 5).

The alignment in Figure 6 for a part of the 2nd intron of *AG* orthologues was generated with CLUSTALX (Jeanmougin et al., 1998) and afterwards corrected by hand. Sequences used other than those of *C. bursa-pastoris* have been published by Hong et al. (2003).

Accession numbers

Sequence Data from this article can be found in the GenBank/EMBL data libraries under accession numbers EU551759-EU551773 and EU662251-EU662266 (see Supplemental Table 6 online).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure 1. DNA blot hybridization experiments with genomic DNA of 1947-wt and 1947-*Spe* plant leaf material**.**

Supplemental Figure 2. MADS domain protein alignment including the isolated members of the *C. bursa-pastoris AG* clade.

Supplemental Figure 3. Phylogeny reconstruction of *AG*-like proteins.

Supplemental Figure 4. Result of PCR experiment testing for a specific deletion in the 2nd intron of *CbpAGa* in the different *Spe* lines.

Supplemental Figure 5. Crossing schedule; overview of crossed performed.

Supplemental Figure 6. Nucleotide alignment of *in situ* hybridization probes of *AG*-like genes

Supplemental Table 1. Test for fertility of 2nd whorl stamens.

Supplemental Table 2. Summary of floral development in *Capsella bursa-pastoris*: Landmark events that occur at beginning of each developmental stage are summarised and assorted in chronological order.

Supplemental Table 3. Segregation patterns of F_3 generations obtained from F_2 plants with questionable intermediate phenotypes of cross 1a.

Supplemental Table 4. Results of phenotyping and genotyping of the parent plants 1947-wt and 1947-*Spe*, the F_1 generation and the F_2 mapping population.

Supplemental Table 5. Sequences of primers used in this work.

Supplemental Table 6. GenBank/EMBL accession numbers of isolated genes.

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FIGURE LEGENDS

Figure 1. Structure of *C. bursa-pastoris* wild-type and *Spe* mutant inflorescences, flowers and floral organs.

(A) Wild-type flower (line 1947-wt).

(B) Flower of a *Spe* variant (line 1947-*Spe*).

(C) Floral diagram of the wild-type. Green, sepals; pink, petals; yellow, stamens; brown, carpels with ovules.

(D) Floral diagram of the *Spe* variant. Organs defined as in **(C)**.

(E) Inflorescence of a 1947-wt plant with open flowers (top view).

(F) Inflorescence of a 1947-*Spe* plant (top view).

(G) From left to right, one lateral and two medial stamens of the 3rd whorl of 1947-wt.

(H) and **(I)** Left side: one lateral and two medial 3rd whorl stamens, right side: 2nd whorl stamens of flowers of **(H)** 1947-*Spe* and of **(I)** 1948-*Spe*.

(J)-(L) Adaxial anther surfaces; **(J)** mature 3rd whorl stamen of 1947-wt; **(K)** 3rd whorl stamen of 1948-*Spe*; **(L)** 2nd whorl stamen of 1948-*Spe*.

(M) Closed anther of 2nd whorl stamen of 1947-*Spe*, arrow: surface area enlarged in **(P)**.

(N) Anther of **(M)** after opening through drought, releasing pollen.

(O) Left: typical anther of 2nd floral whorl of 1947-*Spe*; right: weak 1947-*Spe* phenotype of 2nd whorl stamen, arrow: surface area enlarged in **(P)**.

(P) Detail of petal-like surface structure of anther, indicated by arrowheads in **(M)** and **(O)** (SEM picture, 1947-*Spe*).

(Q) Detail of wild-type adaxial petal surface with single epidermal cells enlarged in inset (SEM picture, 1947-wt).

(R) Malformed anther of a 1948-*Spe* flower.

(S) Pollen grains on 3rd whorl stamen of 1947-wt stained with Alexander´s Reagent; viable pollen stains pink to purple.

(T) Like in **(S)**, but using 2nd whorl stamen of 1947-*Spe*.

(U) Intermediate phenotypes of flowers from an F_1 plant of a cross 1947-wt x 1947-*Spe*.

(V) Range of intermediate organ phenotypes of flowers of a cross 1947-wt x 1947-*Spe*; left: staminoid, right: petaloid intermediate organs.

(W) Intermediate phenotypes of flowers from an F_1 plant of a cross 1947-wt x 1948-*Spe*.

Figure 2. Structure and development of *C. bursa-pastoris* wild-type and *Spe* mutant inflorescences, flowers and floral organs, as revealed by SEM. Scale bar: 20 µm.

(A) Wild-type main inflorescence apex.

(B) Mutant main inflorescence apex (1948-*Spe*).

(C) Wild-type flower bud at late stage 11, arrow points to young petal.

(D) Mutant flower bud at stage 9, arrow points to young 2nd whorl stamen (1947-*Spe*).

Figure 3. *In situ* expression analysis in longitudinal sections of developing flowers of *C. bursa-pastoris*. All sections hybridised with antisense probes if not indicated otherwise. Arrows indicate developing 2nd whorl organs. Scale bar: 100 µm, st: stamen.

(A) and **(B)** *CbpH4* : stage 8 flowers showing the typical punctuate expression pattern of *H4* homologs; **(A)** 1947-wt flower; **(B)** 1947-*Spe* flower.

(C) *CbpAP3*: 1947-wt flower at stage 3 showing expression signals in the area between the sepals and the central dome.

(D) Stage 2 1947-*Spe* flower showing the onset of *CbpAP3* expression in a ring shaped area between centre and margin of flower primordium.

(E) *CbpAP3*: stage 8 1947-wt flower showing later expression in young petal primordia of the 2nd whorl and in stamens of the 3rd whorl.

(F) *CbpAP3*: stage 8 1947-*Spe* flower with signal visible in the stamen primordia of the 2nd whorl, stamens of the 3rd whorl and weakly in the carpel.

(G) *CbpAP3: s*tage 10 1947-wt flower showing late expression in the petals in the 2nd whorl (arrows).

(H) Stage 10 1947-*Spe* flower showing late expression of *CbpAP3* in 3rd and 4th whorl organs and stronger expression in the developing 2nd whorl stamens.

(I) *CbpPI* expression in stage 6 1947-wt flower in stamen primordia.

(J) *CbpPI* expression in stage 3 (left) and stage 4 (right) 1947-*Spe* flowers, both showing signals in area where 2nd and 3rd whorl organs will develop.

(K) and **(L)** Inflorescence apex with *CbpAP1* expression signal in stage 1 and 2 flower primordia **(K)** in 1947-wt plant and **(L)** in 1947-*Spe* plant.

(M) *CbpAP1* sense control of inflorescence apex of 1947-wt plant.

(N) Stage 4 floral bud of 1947-wt flower with *CbpAP1* signal in sepal primordia and adjacent area.

(O) and **(P)** Stage 6 flower without *CbpAP1* expression signal in **(O)** 1947-wt plant and **(P)** 1947-*Spe* plant.

Figure 4. *In situ* analysis of *CbpAG* expression in longitudinal sections of developing flowers of wild-type and *Spe* variant. All sections hybridised with antisense probe if not indicated otherwise. Arrows pointing to 2nd whorl organs. Scale bar: 100 µm.

(A) Late stage 4 flower of 1947-wt plant, expression is visible in central dome of the floral meristem.

(B) Early stage 3 flower of 1947-*Spe* plant, onset of *CbpAG* expression is visible in central dome of the floral meristem.

(C) Early stage 3 flower of 1947-*Spe* plant, hybridization with *CbpAG* sense negative control.

(D) Stage 6 flower of 1947-wt plant, expression visible in whorls three and four.

(E) Stage 6 flower of 1947-*Spe* plant, expression visible in the 3rd and 4th whorl and in young 2nd whorl organ primordia (arrow).

(F) Stage 6 1947-*Spe* flower, sense control.

(G) Stage 8 flower of 1947-wt plant, weak expression in 3rd and 4th whorl organs visible.

(H) Stage 7 flower of 1947-*Spe* plant, expression in 2nd, 3rd and 4th whorl organs.

(I) Stage 8 1947-*Spe,* sense control.

(J) Stage 10 flower of 1947-wt plant, expression visible in the developing stigmatic papillae, style tip and nectary; weak expression in ovules and filaments.

(K) Stage 9 flower of 1947-*Spe* plant, expression visible in developing carpel tip and ovules, in stamens and anthers of 2nd and 3rd whorl stamens.

(L) Stage 9 1947-*Spe,* sense control.

Figure 5. *In situ* analysis of *CbpSTK, CbpSHP1 and CbpSHP2* expression in longitudinal sections of developing flowers and fruits of wildtype and *Spe* variant. All sections hybridised with antisense probe if not indicated otherwise. Scale bar: 100 µm; p: placental tissue; e: endothelium; arrows pointing to 2nd whorl organs.

(A) *CbpSTK*: stage 7 flower of 1947-wt plant, expression signal is visible in the developing carpel and stamens.

(B) *CbpSTK*: stage 6 flower of 1947-*Spe* plant, expression signal visible in 3rd and 4th whorl organs.

(C) *CbpSTK*: stage 6 1947-*Spe* sense control.

(D) *CbpSTK*: stage 9 flower of 1947-wt plant, no expression signal visible.

(E) *CbpSTK*: stage 9 flower of 1947-*Spe* plant, expression signal visible in developing carpel, 2nd and 3rd whorl stamens.

(F) *CbpSTK*: stage 10 1947-*Spe* sense control.

(G) *CbpSTK*: stage 11 flower of 1947-wt plant, expression visible exclusively in the ovules.

(H) *CbpSTK*: stage 11 flower of 1947-*Spe* plant, expression visible exclusively in the ovules.

(I) *CbpSTK*: stage 10 1947-*Spe* sense control.

(J)-(L) No expression signal of *CbpSHP1* and *CbpSHP2* in longitudinal sections of young floral buds; **(J)** *CbpSHP1*: stage 8, 1947-*Spe*; **(K)** *CbpSHP2*: stage 6, 1947-*Spe*; **(L)** *CbpSHP2*: stage 8, 1947-*Spe*.

(M) Cross section of stage 6 flower with *CbpSHP2* expression signal in the placental area of the carpel where ovules will develop, weak staining of stamens and carpel walls represents unspecific background signal, 1947-*Spe*.

(N) Cross section of fertilised young 1947-*Spe* ovule with *CbpSHP2* expression in the endothelium tissue.

(O) Cross section of a fertilised young fruit of a 1947-wt plant, expression of *CbpSHP1* visible at the valve margins.

(P) Enlarged detail of box in **(O)** with *CbpSHP1* expression in the valve margin visible.

(Q) Sense control with a 1947-*Spe* fruit.

Figure 6. Alignment of a 2nd intron section of *AG* orthologs in Brassicaceae, showing a 22 bp deletion combined with three substitutions in *CbpAGa* of the 1947-*Spe* variant in *C. bursa-pastoris*.

Upper part: schematic view of the genomic organisation of the *CbpAG* genomic locus with sequence motifs known from *A. thaliana* (Bao et al. 2004; Hong et al. 2003) indicated; boxes: exons; filled boxes: coding sequence; empty boxes: UTR; connecting lines: non coding sequence; a, j, k: LFY/WUS-binding site; b, d, e: BLR-binding site; c, l: CCAAT-box; f, h: LFY-binding site; g: AGAAT-box; i, m: CArG-box. Lower part alignment section with *CbpAGa* highlighted.

Figure 7. Modified ABC models for the specification of organ identity in the flowers of *C. bursa-pastoris* wild-type and *Spe* plants.

TABLES

Table 1. Genetic analysis of the number and nature of the *Spe*-inducing loci. Segregation patterns of visually analysed phenotypes of F_2 generations originating from the listed F_0 parental lines crossed.

Cross No.	F_0 Parents	F_2 Phenotypes				Model	χ^2	P-value
		Wild -type	Inter-mediate and *Spe*	Inter-mediate	*Spe*			
1	1947-*Spe* x 1947-wt	42	125			1:3	0,002	0,96
1a		42		92	33	1:2:1	2,701	0,26
1b*	1947-*Spe* x 1947-wt *	42		85#	40#	1:2:1	0,102	0,95
2	1947-wt x 1947-*Spe*	42	124			1:3	0,008	0,93
2		42		87	37	1:2:1	0,687	0,71
3	1948-*Spe* x 1947-wt	37	118			1:3	0,105	0,75
3		37		85	33	1:2:1	1,658	0,44
4	1947-wt x 1948-*Spe*	39	115			1:3	0,007	0,93
4		39		75	40	1:2:1	0,117	0,94

* Segregation pattern after test for homozygosity of candidate *Spe* plants in questionable intermediate phenotypes (see Supplemental Table 3 online).
# corrected number after test for homozygosity.

Table 2. Test for allelism of 1947-*Spe* and 1948-*Spe*.

2.1. Initial crosses of the two different *Spe* lines and observed phenotypes.

Initial crosses		Possible F_1 genotype models		Observed phenotype
		Different loci	Same locus	
Spe/Spe**; 1947 x *Spe/Spe*, 1948	Cross **A**	*Spe*/+*, *Spe/+*	*Spe*/Spe*	100% *Spe*
Spe/Spe, 1948 x *Spe*/Spe**; 1947	Cross **B**	*Spe/+*, *Spe*/+*	*Spe/Spe**	100% *Spe*

2.2. Analysis of the F_2 generations resulting from selfing of initial cross offspring F_1 (see above) and resulting from the backcrosses of F_1 members with 1947-wt plants.

	Possible segregation models	Observed phenotypes

Selfing or backcross with offspring of initial cross (Table 3)	(genotypes) Different loci	Same locus	Wild-type	Intermediate	*Spe*
1: F1 Cross **A** selfed	1 : 15#	*Spe*/Spe* *Spe/Spe* *Spe*/Spe**	-	-	105
2: F_1 Cross **A** x 1947-wt	1 : 1 : 1 : 1x	*Spe*/+*, *Spe/+*	-	175	-
3: 1947-wt x F1 Cross **A**	1 : 1 : 1 : 1x	*Spe*/+*, *Spe/+*	-	180	-
4: F1 Cross **B** selfed	1 : 15#	*Spe*/Spe* *Spe/Spe* *Spe*/Spe**	-	-	90
5: 1947-wt x F1 Cross **B**	1 : 1 : 1 : 1x	Spe/+, Spe*/+	-	176	-
6: F1 Cross **B** x 1947-wt	1 : 1 : 1 : 1x	Spe/+, Spe*/+	62	83	-
7: F1 Cross **B** x 1947-wt	1 : 1 : 1 : 1x	Spe/+, Spe*/+	-	43	-
8: F1 Cross **B** x 1947-wt	1 : 1 : 1 : 1x	Spe/+, Spe*/+	-	188	-

* *Spe* locus originating from line 1947-*Spe* background

1 (+/+, +/+) **:** **15** (*Spe*/Spe**, *Spe/Spe*; *Spe*/Spe**, *Spe* /+; *Spe*/Spe**, +/+; *Spe*/+*, *Spe/Spe*; *Spe*/+*, *Spe/+*; Spe*/+, +/+; +/+, Spe/+)

x **1** (+/+, +/+) **: 1** (Spe*/+, +/+) **: 1** (+/+ , Spe/+) **: 1** (Spe*/+, Spe/+)

Table 3. Results of pyrosequencing analysis for co-segregation of wild-type phenotypes of the segregating F_2 population with the *AG*-like gene SNPs (genotypes) of the wild-type F_0 parent plants.

Gene	SNP (genotype)			Number of investigated wild-type plants	Plants homozygous for the wild-type SNP	Percentage of co-segregation
	Wild-type homo-zygous	*Spe* homo-zygous	hetero-zygous			
CbpAGa	C/C	-/-	C/-	9	9	100 %
CbpAGb	A/A	C/C	A/C	9	3	33,3 %
CbpSHP1a	T/T	G/G	T/G	9	4	44,4 %
CbpSHP1b	A/A	C/C	A/C	9	2	22,2 %
CbpSHP2a	G/G	T/T	G/T	9	1	11,1 %
CbpSHP2b	T/T	A/A	T/A	9	2	22,2 %
CbpSTKa	T/T	G/G	T/G	11	5	45,5 %
CbpSTKb	T/T	C/C	T/C	9	2	22,2 %

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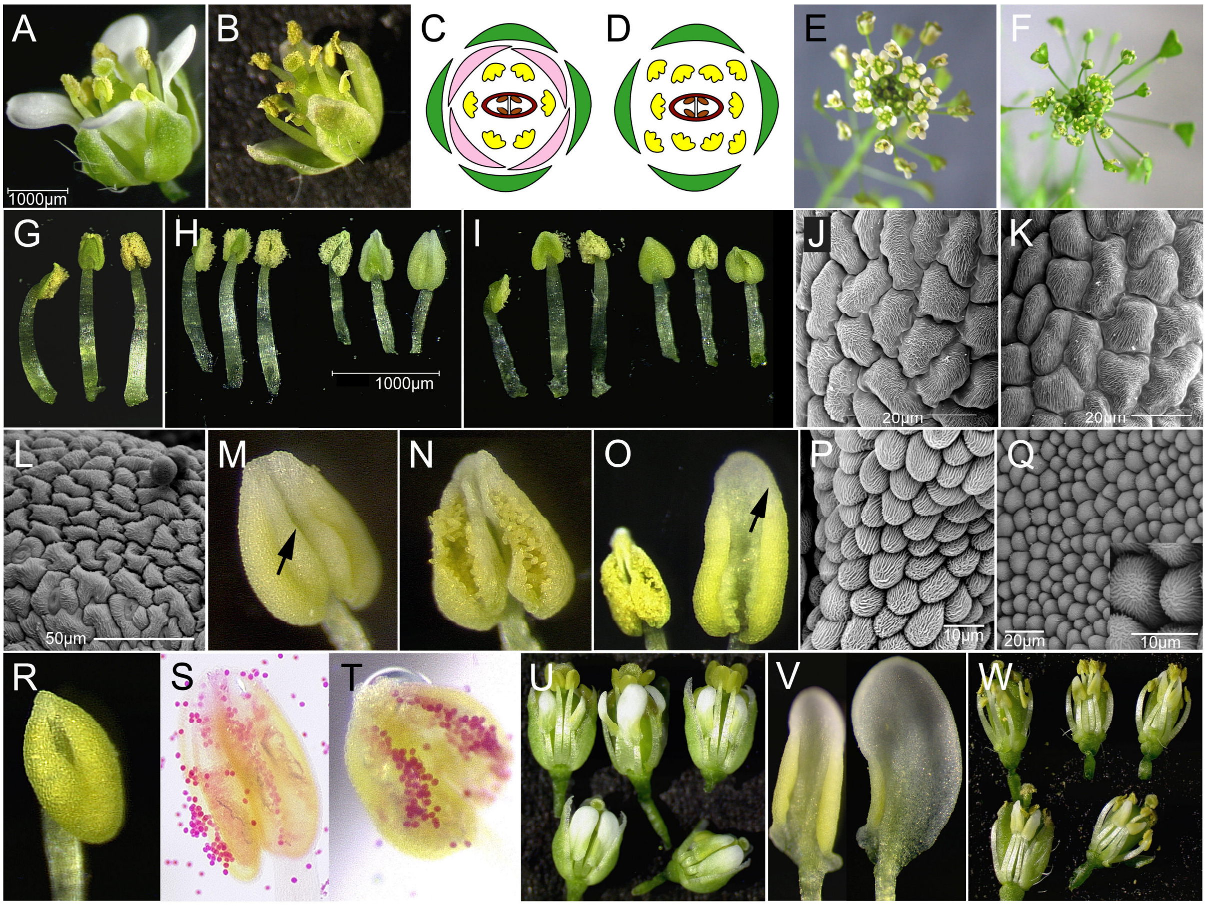


Figure 1. Structure of *C. bursa-pastoris* wild-type and Spe mutant inflorescences, flowers and floral organs.

(A) Wild-type flower (line 1947-wt).
(B) Flower of a Spe variant (line 1947-*Spe*).
(C) Floral diagram of the wild-type. Green, sepals; pink, petals; yellow, stamens; brown, carpels with ovules.
(D) Floral diagram of the *Spe* variant. Organs defined as in **(C)**.
(E) Inflorescence of a 1947-wt plant with open flowers (top view).
(F) Inflorescence of a 1947-*Spe* plant (top view).
(G) From left to right, one lateral and two medial stamens of the 3rd whorl of 1947-wt.
(H) and **(I)** Left side: one lateral and two medial 3rd whorl stamens, right side: 2nd whorl stamens of flowers of **(H)** 1947-*Spe* and of **(I)** 1948-*Spe*.
(J)-**(L)** Adaxial anther surfaces; **(J)** mature 3rd whorl stamen of 1947-wt; **(K)** 3rd whorl stamen of 1948-Spe; **(L)** 2nd whorl stamen of 1948-*Spe*.
(M) Closed anther of 2nd whorl stamen of 1947-*Spe*, arrow: surface area enlarged in **(P)**.
(N) Anther of **(M)** after opening through drought, releasing pollen.
(O) Left: typical anther of 2nd floral whorl of 1947-*Spe*; right: weak 1947-*Spe* phenotype of 2nd whorl stamen, arrow: surface area enlarged in **(P)**.
(P) Detail of petal-like surface structure of anther, indicated by arrowheads in **(M)** and **(O)** (SEM picture, 1947-*Spe*).
(Q) Detail of wild-type adaxial petal surface with single epidermal cells enlarged in inset (SEM picture, 1947-wt).
(R) Malformed anther of a 1948-*Spe* flower.
(S) Pollen grains on 3rd whorl stamen of 1947-wt stained with Alexander´s Reagent; viable pollen stains pink to purple.
(T) Like in **(S)**, but using 2nd whorl stamen of 1947-*Spe*.
(U) Intermediate phenotypes of flowers from an F1 plant of a cross 1947-wt x 1947-*Spe*.
(V) Range of intermediate organ phenotypes of flowers of a cross 1947-wt x 1947-*Spe*; left: staminoid, right: petaloid intermediate organs.
(W) Intermediate phenotypes of flowers from an F1 plant of a cross 1947-wt x 1948-*Spe*.

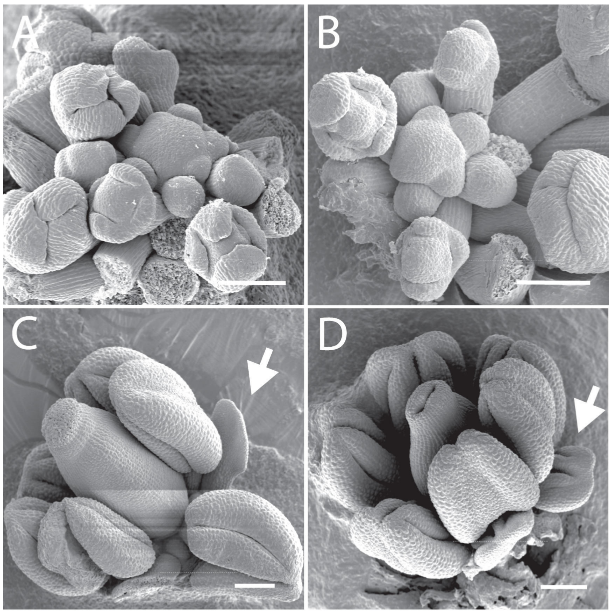


Figure 2. Structure and development of *C. bursa-pastoris* wild-type and *Spe* mutant inflorescences, flowers and floral organs, as revealed by SEM. Scale bar: 20 µm.

(A) Wild-type main inflorescence apex.
(B) Mutant main inflorescence apex (1948-*Spe*).
(C) Wild-type flower bud at late stage 11, arrow points to young petal.
(D) Mutant flower bud at stage 9, arrow points to young 2nd whorl stamen (1947-*Spe*).

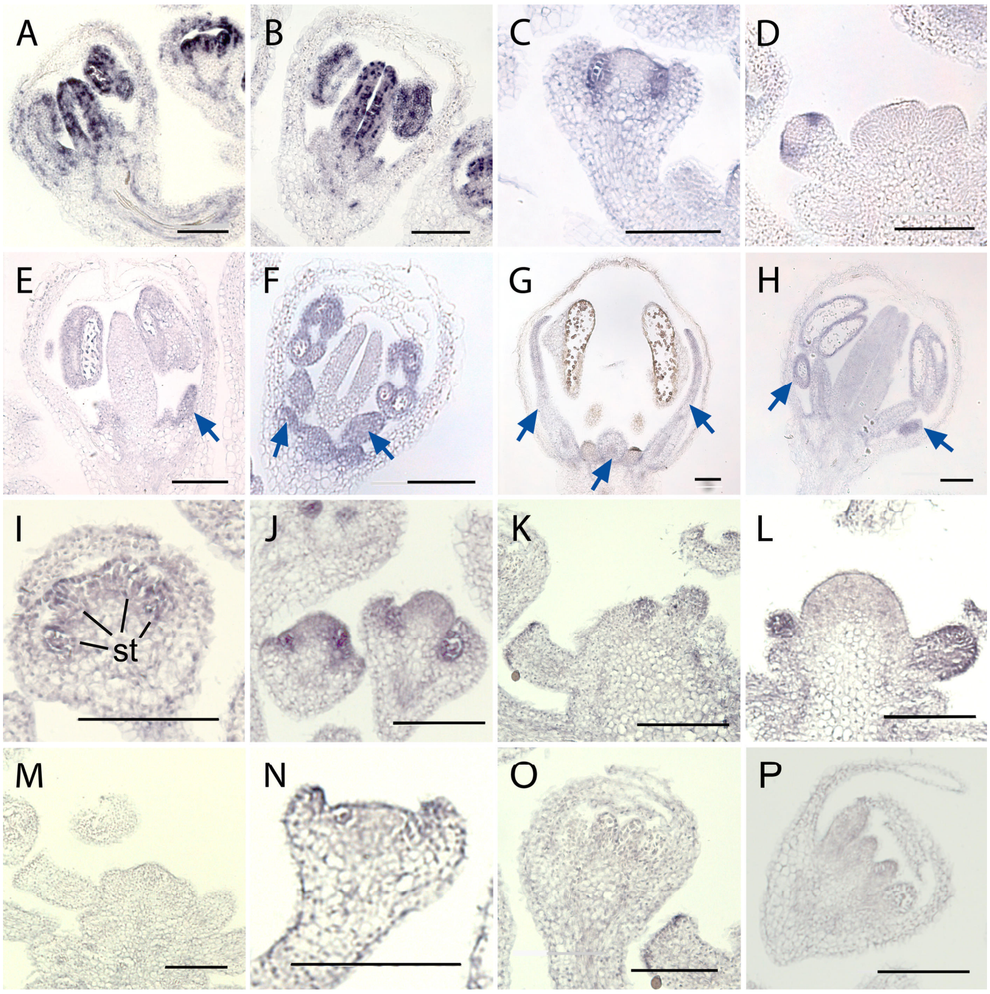


Figure 3. *In situ* expression analysis in longitudinal sections of developing flowers of *C. bursa-pastoris*. All sections hybridised with antisense probes if not indicated otherwise. Arrows indicate developing 2nd whorl organs. Scale bar: 100 µm, st: stamen.

(A) and **(B)** *CbpH4* : stage 8 flowers showing the typical punctuate expression pattern of *H4* homologs; **(A)** 1947-wt flower; **(B)** 1947-*Spe* flower.
(C) *CbpAP3*: 1947-wt flower at stage 3 showing expression signals in the area between the sepals and the central dome.
(D) Stage 2 1947-*Spe* flower showing the onset of *CbpAP3* expression in a ring shaped area between centre and margin of flower primordium.
(E) *CbpAP3*: stage 8 1947-wt flower showing later expression in young petal primordia of the 2nd whorl and in stamens of the 3rd whorl.
(F) *CbpAP3*: stage 8 1947-Spe flower with signal visible in the stamen primordia of the 2nd whorl, stamens of the 3rd whorl and weakly in the carpel.
(G) *CbpAP3*: stage 10 1947-wt flower showing late expression in the petals in the 2nd whorl (arrows).
(H) Stage 10 1947-*Spe* flower showing late expression of *CbpAP3* in 3rd and 4th whorl organs and stronger expression in the developing 2nd whorl stamens.
(I) *CbpPI* expression in stage 6 1947-wt flower in stamen primordia.
(J) *CbpPI* expression in stage 3 (left) and stage 4 (right) 1947-*Spe* flowers, both showing signals in area where 2nd and 3rd whorl organs will develop.
(K) and **(L)** Inflorescence apex with *CbpAP1* expression signal in stage 1 and 2 flower primordia **(K)** in 1947-wt plant and **(L)** in 1947-*Spe* plant.
(M) *CbpAP1* sense control of inflorescence apex of 1947-wt plant.
(N) Stage 4 floral bud of 1947-wt flower with *CbpAP1* signal in sepal primordia and adjacent area.
(O) and **(P)** Stage 6 flower without *CbpAP1* expression signal in **(O)** 1947-wt plant and **(P)** 1947-*Spe* plant.

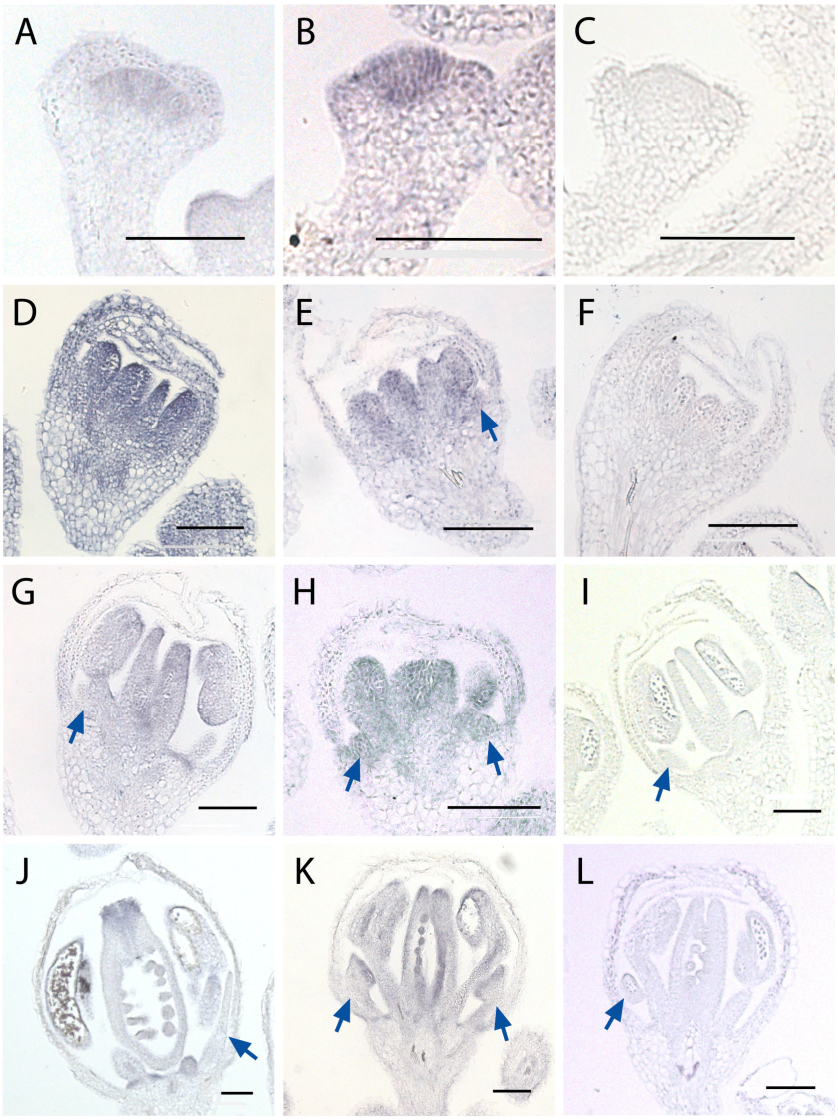


Figure 4. *In situ* analysis of *CbpAG* expression in longitudinal sections of developing flowers of wild-type and *Spe* variant. All sections hybridised with antisense probe if not indicated otherwise. Arrows pointing to 2nd whorl organs. Scale bar: 100 µm.

(A) Late stage 4 flower of 1947-wt plant, expression is visible in central dome of the floral meristem.
(B) Early stage 3 flower of 1947-*Spe* plant, onset of *CbpAG* expression is visible in central dome of the floral meristem.
(C) Early stage 3 flower of 1947-*Spe* plant, hybridization with *CbpAG* sense negative control.
(D) Stage 6 flower of 1947-wt plant, expression visible in whorls three and four.
(E) Stage 6 flower of 1947-*Spe* plant, expression visible in the 3rd and 4th whorl and in young 2nd whorl organ primordia (arrow).
(F) Stage 6 1947-*Spe* flower, sense control.
(G) Stage 8 flower of 1947-wt plant, weak expression in 3rd and 4th whorl organs visible.
(H) Stage 7 flower of 1947-*Spe* plant, expression in 2nd, 3rd and 4th whorl organs.
(I) Stage 8 1947-*Spe*, sense control.
(J) Stage 10 flower of 1947-wt plant, expression visible in the developing stigmatic papillae, style tip and nectary; weak expression in ovules and filaments.
(K) Stage 9 flower of 1947-*Spe* plant, expression visible in developing carpel tip and ovules, in stamens and anthers of 2nd and 3rd whorl stamens.
(L) Stage 9 1947-*Spe*, sense control.

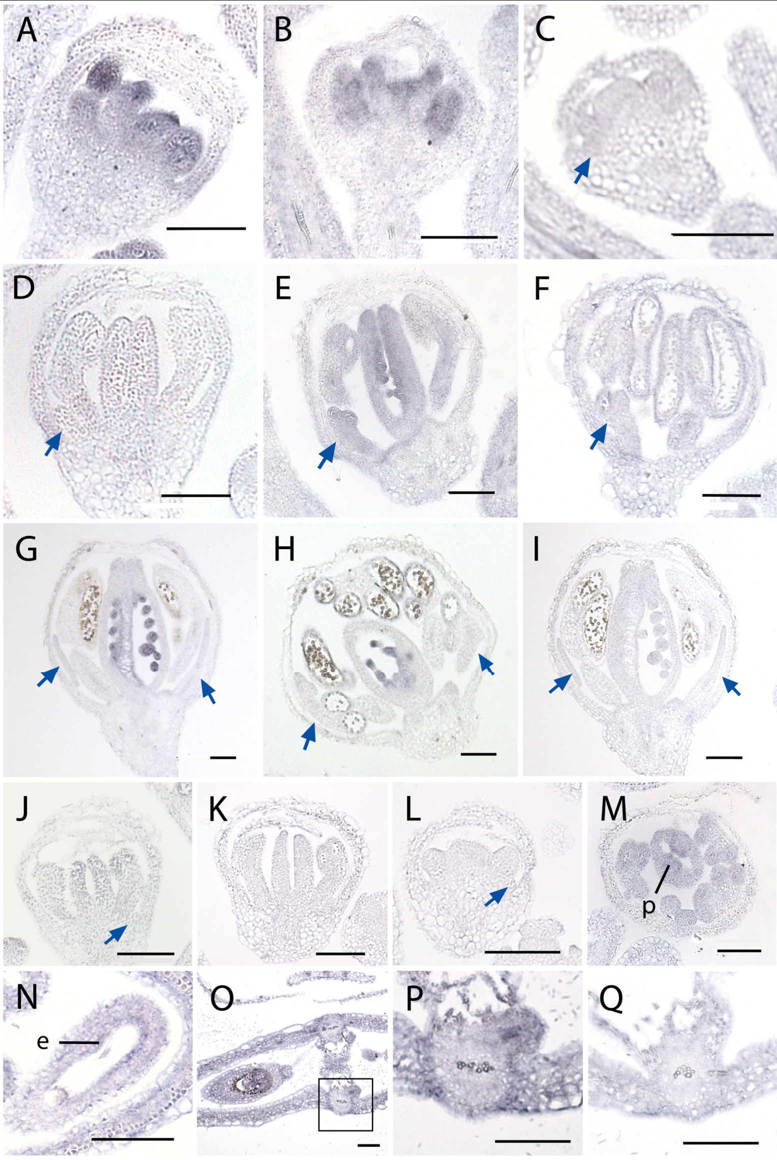
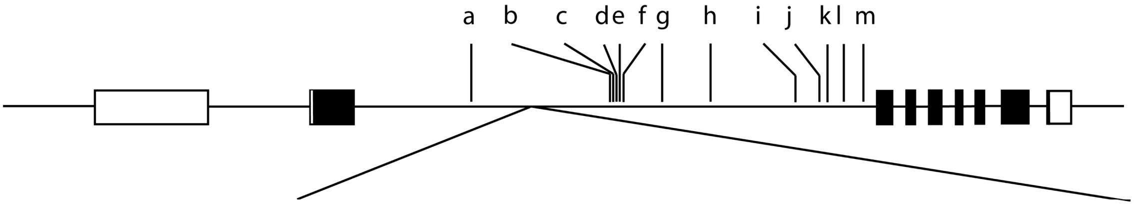


Figure 5. *In situ* analysis of *CbpSTK*, *CbpSHP1* and *CbpSHP2* expression in longitudinal sections of developing flowers and fruits of wildtype and *Spe* variant. All sections hybridised with antisense probe if not indicated otherwise. Scale bar: 100 µm; p: placental tissue; e: endothelium; arrows pointing to 2nd whorl organs.

(A) *CbpSTK*: stage 7 flower of 1947-wt plant, expression signal is visible in the developing carpel and stamens.
(B) *CbpSTK*: stage 6 flower of 1947-*Spe* plant, expression signal visible in 3rd and 4th whorl organs.
(C) *CbpSTK*: stage 6 1947-*Spe* sense control.
(D) *CbpSTK*: stage 9 flower of 1947-wt plant, no expression signal visible.
(E) *CbpSTK*: stage 9 flower of 1947-*Spe* plant, expression signal visible in developing carpel, 2nd and 3rd whorl stamens.
(F) *CbpSTK*: stage 10 1947-*Spe* sense control.
(G) *CbpSTK*: stage 11 flower of 1947-wt plant, expression visible exclusively in the ovules.
(H) *CbpSTK*: stage 11 flower of 1947-*Spe* plant, expression visible exclusively in the ovules.
(I) *CbpSTK*: stage 10 1947-*Spe* sense control.
(J)-(L) No expression signal of *CbpSHP1* and *CbpSHP2* in longitudinal sections of young floral buds; **(J)** *CbpSHP1*: stage 8, 1947-*Spe*; **(K)** *CbpSHP2*: stage 6, 1947-*Spe*; **(L)** *CbpSHP2*: stage 8, 1947-*Spe*.
(M) Cross section of stage 6 flower with *CbpSHP2* expression signal in the placental area of the carpel where ovules will develop, weak staining of stamens and carpel walls represents unspecific background signal, 1947-*Spe*.
(N) Cross section of fertilised young 1947-*Spe* ovule with *CbpSHP2* expression in the endothelium tissue.
(O) Cross section of a fertilised young fruit of a 1947-wt plant, expression of *CbpSHP1* visible at the valve margins.
(P) Enlarged detail of box in **(O)** with *CbpSHP1* expression in the valve margin visible.
(Q) Sense control with a 1947-*Spe* fruit.

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Raphanus sativus            TTATTTATTTTGTCGTTATGGAAAA-----ATGACAACTGTGTTTAAGC----TTAGATTAGTT
Cakile maritima             TTATTAATTTTGTAGTTAGTATGGAA---AATATCAAATATCTTTCGGC----TTAGATTAGTT
Brassica oleracea           TTATTTATTTTGTAGTTATGGAAA------ATATCAAATATCTTTGAGC----TTAGATTAGTT
Diplotaxis catholica        TTATTTCTTTTGTTGTTATGGAAAG-----ATGTCAACTAACTTTAAGC----TTAGATTAGTT
Thlaspi arvense             CTTTTTATTTGGTTGTTATTGAAAA----------ACATA--TATGAGC----TAGGATTAGTT
Conringia orientalis        TTATTTATTTTGTAGTTGTGGAAAA----------ATATAGATATGAGC----TAGGATTAGTT
Thysanocarpus               TTATTTAGTTTGTAGTTATTGTGTAA--AAATATAAAATATCTATGAGT----TGGGATTAGTT
Guillenia flavescens 2      TTATTTAGTTTGTAGTTATTGTGGGA--ATATATCTAATATCTATGAGC----TGGGATTAGTT
Guillenia flavescens 1      TTATTTGTTTTGTAGTTATGGAAAAA--TTTTCAAATATCTA--TGAGCC---TGGGATAAGTT
Streptanthus glandulosus    TTATTTGTTTTGTAGTTATGGAAAA---TTTTCAAATATCTATATGAGCG---TGGGATAAGTT
Lepidium phlebopetalum      AGTGTTATTTTGTAGTTTTGTAGAA------------AAATGTATGAGT----TATGATTAGGT
Arabis gunnisoniana         AGAGTCATTTTATAGTTTTGTAAAA------------ACATATATGAGC----TAGGATTAGTT
Camelina sativa             AGAGTCATTTTGTAGTTTTGGAAAT------------ACACATATGAGGTA--CAGGATTAGTT
Olimarabidopsis pumila      AGGTTTATTTTGTAGTTTTTGGAAAA-----------ATATATATGAGCTAGCTAGGATTAGTT
Erysimum cheiri             AGGGTTATTTTGTAGTTTTGGAAAAA------------TATATATGAGC----TAAGATTAGTT
Arabidopsis arenosa         TTATTAATTTTGTAGTTTTGGAAAA--------GTATATATATATGAGC----TAAGATTAGTT
Arabidopsis lyrata          TTATT---TTTGTAGTTTTCGAAAA--------GTATATATA--TGAGC----TAAGATTAGTT
Arabidopsis thaliana        TTATT---TTTGTAGTTTTGGAAAA------------TTATATATGAGC----TAAGATTAGTT
Capsella rubella            AGAGTCATTTTGTAGTTTTGGAAA--------------AAAAAATG-------------TAGTT
CbpAGa_1947-Spe             AGAGTCATTC----------------------------AAAGTATGAGCTA--TAGGATTAGTT
CbpAGa_1947-wt              AGAGTCATTTTGTAGTTTTGGAAAGA------AAAAAATATGTATGAGCTA--TAGGATTAGTT
CbpAGb 1947-wt              -GAGTCATTTTGTAGTTTTGGAAATATATATATATATATATATATGAGCT----ATGATTAGTT
```

Figure 6. Alignment of a 2nd intron section of *AG* orthologs in Brassicaceae, showing a 22 bp deletion combined with three substitutions in *CbpAGa* of the 1947-*Spe* variant in *C. bursa-pastoris*.
Upper part: schematic view of the genomic organisation of the *CbpAG* genomic locus with sequence motifs known from *A. thaliana* (Bao et al. 2004; Hong et al. 2003) indicated; boxes: exons; filled boxes: coding sequence; empty boxes: UTR; connecting lines: non coding sequence; a, j, k: LFY/WUS-binding site; b, d, e: BLR-binding site; c, l: CCAAT-box; f, h: LFY-binding site; g: AGAAT-box; i, m: CArG-box. Lower part alignment section with *CbpAGa* highlighted.

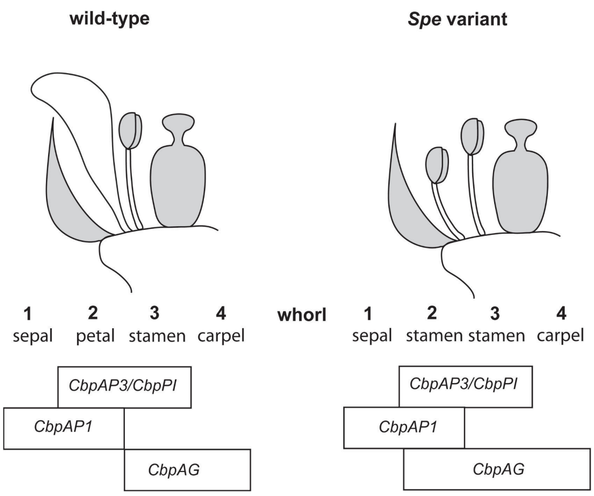

Figure 7. Modified ABC models for the specification of organ identity in the flowers of *C. bursa-pastoris* wild-type and *Spe* plants.

5 Manuskript III

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Germline transformation of Shepherd's purse (*Capsella bursa-pastoris*) by the 'floral dip' method as a tool for evolutionary and developmental biology

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Abstract

Capsella bursa-pastoris is an attractive model system for evolutionary and developmental biology. To facilitate future studies on gene function, the 'floral dip' method was adapted to achieve germline transformation of *C. bursa-pastoris*. The *GFP* and *BASTA-resistance* (*BAR* ^{r}) genes were used as markers for screening or selecting, respectively, putative transgenic *C. bursa-pastoris* plants and the β-glucuronidase (*GUS*) gene as well as the *GFP* gene for monitoring transgene expression level. We tested two *Agrobacterium* strains, LBA4404 and GV3101, for their ability to transform *C. bursa-pastoris*. In contrast to *Arabidopsis thaliana*, for which both strains were able to transform different ecotypes, only GV3101 gave satisfactory transformation rates with *C. bursa-pastoris*. Furthermore, we evaluated the effects of different concentrations of sucrose and the surfactant Silwet L-77 on the efficiency to generate transgenic *C. bursa-pastoris* plants and identified an efficient medium containing 10% (w/v) sucrose and 0.02–0.05% (v/v) Silwet L-77. Using Southern hybridisation, we confirmed the integration of the marker gene in the plant genome and the stable heredity of the introduced genes in the next generation.

Keywords: *Agrobacterium*-mediated transformation; Floral-dipping; *GUS*-expression; Herbicide resistance; *Capsella*; Transgenic Shepherd's purse

1. Introduction

Shepherd's purse, *Capsella bursa-pastoris* (L.) Medik., is a close relative of the genetic model plant *Arabidopsis thaliana* (Koch and Kiefer, 2005) and belongs to the five most widespread flowering plants on our planet (Hurka et al., 2003). It is a member of a small genus within the mustard family (Brassicaceae) which may only comprise three species; these, however, show remarkable differences in ploidy level, breeding systems and habitat range (Zunk et al., 1999; Hurka et al., 2005). Two of the *Capsella* species, *C. grandiflora* and *C. rubella*, are diploid, while the third one, *C. bursa-pastoris*, is tetraploid. *C. bursa-pastoris* is predominantly selfing, and is distributed mainly in disturbed, 'man-made' habitats, almost all over the world (Hurka and Neuffer, 1997). Concerning reproductive biology and ploidy level, comparison with outgroup species strongly suggests that *C. grandiflora* represents the most ancestral and *C. bursa-pastoris* the most derived character states (Hurka et al., 2005).

Within *Capsella*, *C. bursa-pastoris* is of special interest. It is a classical model plant for investigations on the early development of eudicot embryos (Schulz and Jensen, 1968). Moreover, *C. bursa-pastoris* shows some interesting and potentially

Abbreviations: BAP, Benzylaminopurine; BAR^{r}, Basta-resistance gene; CaMV, Cauliflower Mosaic Virus; *Cbp*AG, *Capsella bursa-pastoris* Agamous; CHSA, Chalcone synthase; DIG, Digoxigenin; GFP, Green fluorescent protein; GUS, β-glucuronidase; MAS, Mannopine synthase; MS, Murashige & Skoog; NPTII, Neomycin phosphotransferase II; NOS, Nopaline synthase; OD, Optical density; pAG7, Agropine synthase polyadenylation signal; SI, Self-incompatibility.

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important morphogenetic phenomena that are, however, quite rare and hence cannot be easily studied elsewhere. In the flowers of the homeotic mutant *Stamenoid petals* (*Spe*), for example, stamens develop in the second floral whorl, where usually petals would occur, while all other floral organs are unaffected (Nutt et al., 2006; Hintz et al., 2006). The *Spe* mutant is interesting from both a developmental and an evolutionary point of view (Nutt et al., 2006; Hintz et al., 2006). Several lines of evidence suggest that homeotic changes played a considerable role during the evolution of flowers, but the relevance of homeotic transformations during the origin of morphological novelties has remained a very controversial topic (reviewed by Theiβen, 2006). Thus *C. bursa-pastoris* is an attractive experimental system for evolutionary and developmental biology.

C. bursa-pastoris is quite easy to cultivate and propagate. Its life cycle is fairly short and allows the growth of two to three generations per year (depending on the variety). And despite being tetraploid, *C. bursa-pastoris* switched already to disomic inheritance during evolution (Hurka and Düring, 1994), which makes crossing experiments easier to interpret. The order, orientation and sequence of genes is very similar in *Arabidopsis* and *Capsella*, with more than 90% sequence identity within exons (Acarkan et al., 2000; Koch and Kiefer, 2005). This allows the identification of genes within *Capsella* with the help of the *Arabidopsis* genome (*Arabidopsis* Genome Initiative, 2000). Due to its close relationship to the model plant *A. thaliana* numerous experimental tools are available to study the genus *Capsella*, and more are being developed. Experimentation along these lines will be further facilitated by the sequencing of the genome of *Capsella rubella* (Joint Genome Institute, United States Department of Energy).

To investigate traits from an ecological, developmental or evolutionary point of view transgenic technology is often extremely helpful. For example, the evolution of the self-incompatibility system in *Arabidopsis* was studied by transfer of two S locus genes from the self-incompatible *A. lyrata* into the self-compatible *A. thaliana* (Nasrallah et al., 2002), and similar experiments may help to investigate the evolution of the SI (self-incompatibility) system that is active in *C. grandiflora* (Paetsch et al., 2006) as well. But so far successful transformation of *Capsella* has not been reported.

In *Capsella*'s close relative *A. thaliana*, genetic transformation became a very simple procedure by development of the 'floral dip' method (Clough and Bent, 1998). 'Floral dip' is an *in planta* method, because genes are delivered into intact plants. Unlike previous methods (reviewed by Bent, 2000), the 'floral dip' procedure requires neither tissue culture nor vacuum infiltration and is hence very labour-efficient. The 'floral dip' method could also be applied to *Arabidopsis lasiocarpa*, radish (*Raphanus sativus ssp. longipinnatus*) and *Medicago truncatula* with good success (Tague, 2001; Curtis and Nam, 2001; Trieu et al., 2000), but similar attempts with *C. bursa-pastoris* failed (Tague, 2001).

The previous studies in *Arabidopsis* and *Raphanus* revealed that the presence of the tri-siloxane 'Silwet L-77' and of sucrose in the inoculation medium as well as the developmental stage of the dipped plants significantly influence the efficiency of transgenic plant production. Here we report a protocol for successfully generating genetically modified *C. bursa-pastoris* plants. Besides the parameters mentioned above we also found that choice of the right strain of *Agrobacterium* is essential for efficient transformation.

2. Materials and methods

2.1. Plant material

C. bursa-pastoris (L.) Medik. selfed offspring was used from individuals of the line 'wt 6/1+2' with normally developed floral whorls, and individuals from line '*Spe* 9/9', a variety where petals in the second floral whorl are replaced by stamens; both lines are from population '1947', growing in Gau-Odernheim (Rheinhessen, Germany). Additional selfed offspring used was from line '*Spe* 2/4', of the population '1948', growing near Warburg (Westfalen, Germany) (Nutt et al., 2006). On the following plant line names will be abbreviated by '1947-*Spe*', '1947-wt' and '1948-*Spe*', respectively. *Capsella* population and plant line numbers refer to the Brassicaceae Germ Plasm Collection of the Department of Systematic Botany, University of Osnabrück, Germany. Generation times differ between the plant lines. From sowing to the start of bolting, line 1947-wt needs 6 weeks, line 1947-*Spe* needs 8 to 12 weeks, and line 1948-*Spe* needs 5 to 6 weeks under our standard growing conditions. All plant lines need 4 weeks from start of flowering to senescence of the inflorescence and ripening of fruits and seeds.

2.2. Plant growth conditions

Plants were cultivated in a mixture of soil-based seedling substrate (Klasmann, Germany), Vermiculite (Klasmann, Germany) and sand (8:1:1 by vol) supplemented with 1 g/l of each Osmocote mini (Scotts, The Netherlands) and Triabon (Compo, Germany) as long-term fertilizer. Prior to germination seeds were stratified for 4 days at 4 °C on top of humid soil surface. Growing conditions were set to a 16-h-photoperiod with 250–300 µmol photons/m^2s at 22 °C and 8h at 18 °C without light, and relative humidity levels of about 50% (day) to 60% (night).

2.3. Bacterial strains and plasmids

The *Agrobacterium tumefaciens* strains GV3101/pMP90 (Koncz and Schell, 1986) and LBA4404 (Hoekema et al., 1983) were used in this study. Each strain carried either the binary vector mGFP5-ER (Haseloff et al., 1997), pGPTV-bar (www.biotech.unl.edu) or pFGC5941 (www.chromdb.org). The T-DNA cassette of mGFP5-ER-vector contains a modified *GFP* gene driven by the CaMV (Cauliflower Mosaic Virus) 35S promoter (Fig. 1a). The additional kanamycin resistance gene *NPTII* of the vector mGFP5-ER has not been used in this study. The vector pGPTV-bar contains the *BASTA-resistance* gene (*BAR* ^{r}) and a *GUS* gene as a second selectable marker, both driven by NOS promoters (Fig. 1b). The vector pFGC5941, which was designed for RNAi experiments, carries the *BAR* ^{r}

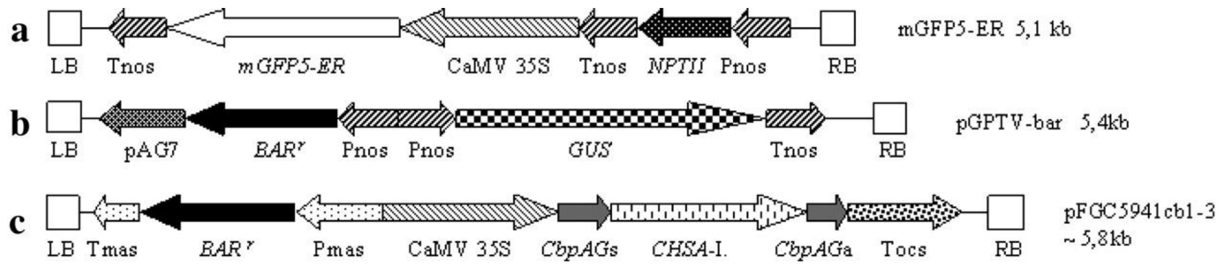

Fig. 1. Linear maps of the different T-DNA cassettes. LB/RB: left/right T-DNA border sequences; (a) mGFP5-ER cassette; Pnos: NOS (nopaline synthase)-promoter; Tnos: NOS terminator; *NPTII*: neomycin phosphotransferase gene; CaMV 35S: 35S-promoter of the Cauliflower Mosaic Virus; *mGFP5-ER*: modified *GFP* (green fluorescent protein) gene. (b) pGPTV-bar cassette; Pnos: NOS promoter; Tnos: NOS terminator; pAG7: agropine synthase-polyadenylation signal; GUS: β-glucuronidase gene; *BAR^r: BASTA-resistance* gene (phosphinotricine acetyltransferase); (c) pFGC5941cb1-3 cassette; Pmas: MAS (mannopine synthase) promoter; Tmas: MAS terminator; Tocs: OCS (octopine synthase) terminator; CaMV 35S: 35S-promoter; *CHSA*-I: *CHSA* (chalcone synthase) intron from *Petunia hybrida*; *CbpAG* a/s: *Capsella bursa-pastoris AGAMOUS* gene fragments in antisense/sense direction; *BAR ^{r}*: BASTA-resistance gene.

gene driven by a MAS promoter as a selective marker. For generation of RNAi constructs the cassette contains the *CHSA* intron flanked by two recognition sites for restriction enzymes for insertion of complementary RNAi fragments of interest, all driven by the CaMV 35S promoter. Here, partial sequences of the *AGAMOUS* orthologue of *C. bursa-pastoris* (*Cpb AG*) were cloned in sense and antisense direction on both sides of the *CHSA* intron yielding vector derivatives pFGC5941cb1-3 (Fig. 1c). All vectors contained a marker conferring kanamycin-resistence for selection in *E. coli*. *Agrobacterium* strain GV3101 was grown on YEB medium containing 50 µg/ml kanamycin and 25 µg/ml gentamycin, LBA4404 on YEB medium with 50 µg/ml kanamycin only. Both strains were cultivated in 1l medium for 24 h at 28 °C for preparation of infiltration media.

2.4. Production of transgenic plants

Transgenic plants were generated using the 'floral dip' method (Clough and Bent, 1998). For *Capsella* plants primary bolts were not clipped prior to inoculation with *Agrobacterium*, but silicules and open flowers were removed from flowering plants. The whole inflorescences of each plant were submerged into a 2 l beaker containing a 24 h culture of *Agrobacterium* which, after centrifugation with 7000 × g for 10 min at 4 °C, was resuspended in the appropriate infiltration medium to an $OD_{600\ nm}$ of approximately 1. Several compounds of the infiltration media were tested, including different concentrations of sucrose, Silwet L-77 and different amounts of Murashige and Skoog (MS) basal medium with the pH adjusted to pH 5.7. In one of the two MS-media benzylaminopurine (BAP, Sigma Aldrich, USA) was additionally added. For each variant approximately ten plants were immersed into the bacterial solution, gently agitated for about 5 s and then removed. Dipped plants were immediately covered with a plastic bag and placed into the dark over night. Bags were then removed and the plants were returned into the light. All plants were dipped again after 7 days. Plants (T_0 generation) were grown under selfing conditions until stem and silicules completely lost their green colour and the seed colour changed to dark brown. Mature seeds were harvested and stored for a fortnight at 4 °C. These seeds (T_1 generation) were sown again and 5 days after germination seedlings were tested for activity of the respective marker genes.

2.5. Visual verification of transgenic seedlings

Expression of *GFP* in 5-day-old seedlings was observed using a fluorescence microscope MZ FL III (Leica) equipped with a GFP 3/plant filter (excitation: 470/40 nm, barrier filter: 525/50 nm). Expression of the *BAR ^{r}* gene was tested by spraying 5-day-old seedlings with 0.1% BASTA-solution (Aventis) on two following days. In both cases transgenic plants were transplanted into individual pots, grown to maturity and seeds were harvested.

For the GUS staining assay individual leaves from 4 to 8 week old BASTA-resistant plants were incubated in 0.1M sodium phosphate (pH 7.4), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.5% (v/v) Triton X-100, 0.5 mg/ml X-Gluc in DMSO for 1 week at 37 °C. Afterwards the leaves were washed in 70% ethanol until all chlorophyll had been removed.

2.6. PCR based verification of GFP gene integration

Genomic DNA was extracted from leaves of randomly chosen plants transformed with mGFP5-ER and, as a negative control, non-transformed plants with the CTAB method (Rogers and Bendich, 1988). PCR analysis was performed using the following two primers: 5′-CATTTCATTTGGAGAGAACACG-3′ and 5′-GATAATCATCGCAAGACCGG-3′. The expected length of the amplified product is 941 bp. The applied PCR program involved 30 cycles of 94 °C (30 s), 54 °C (30 s) and 72 °C (1 min) in an Eppendorf Mastercycler.

2.7. Southern blot analysis of transgenic plants

To determine the copy number of the transgenes in the genome of *C. bursa-pastoris*, total DNA was extracted from leaves of plants of interest. 20 µg of DNA was digested with *Eco*RI or *Hin*dIII, separated on 1% (w/v) agarose gels and blotted onto positively charged nylon membranes (VWR, Germany). Membranes were hybridized with digoxigenin (DIG)-labelled *BAR ^{r}* or *GFP* gene fragments according to manufacturer's instructions (Roche Diagnostics, Germany). The hybridization probes were prepared from the pGPTV-bar or mGFP5-ER plasmids, respectively, by PCR. In both cases partial gene sequences were used. To amplify the *BAR ^{r}* gene fragment the primers 5′-CTGAAGTCCAGCTGCCAG-3′ and 5′-GAGACAAGCACGGTCAACT-3′

Table 1
Overview of tested plant lines and vectors, demonstrating the effects of different compositions of infiltration media on the production of transformed seeds of plants dipped with the *Agrobacterium* strain GV3101

Infiltration medium variants	Vector used	Plant line		
		1947-wt	1947-*Spe*	1948-*Spe*
1 3% sucrose, 0.05% Silwet-L77, 1x MS-salts, pH 5.7	mGFP5-ER [a]	2/8320 = 0.02% [d]	1/14735 = 0.01%	
	pGPTV-bar [b]	2/717 = 0.28%	1/8485 = 0.01%	
2 5% sucrose, 0.05% Silwet-L77	mGFP5-ER	0/7632 = 0	1/4525 = 0.02%	
	pGPTV-bar	2/5102 = 0.04%	0/3920 = 0	
	pFGC5941 [c]	7/25,080 = 0.03%	0/8250 = 0	
		7/39,800 = 0.02%	2/22,800 = 0.01%	
3 5% sucrose, 0.005% Silwet-L77, 1/2xMS-salts, 44 mM BAP, pH 5.7	mGFP5-ER	0/18,090 = 0	0/2555 = 0	
	pGPTV-bar	2/5900 = 0.03%	2/9604 = 0.02%	
4 5% sucrose, 0.02% Silwet-L77	mGFP5-ER	7/5612 = 0.13%	3/5965 = 0.05%	
	pGPTV-bar	4/1276 = 0.31%	2/2553 = 0.08%	
		3/2984 = 0.10%		
	pFGC5941	1/2460 = 0.04%		
		2/11,742 = 0.017%		
		2/4035 = 0.05%		
5 10% sucrose, 0.05% Silwet-L77	pGPTV-bar	14/3493 = 0.40%		4/2922 = 0.14%
6 10% sucrose, 0.02% Silwet-L77	mGFP5-ER	4/6655 = 0.06%		0/18,240 = 0
	pFGC5941	1/2475 = 0.04%		44/16,710 = 0.26%
		13/2703 = 0.48%		0/13,020 = 0
		6/2574 = 0.23%		1/8940 = 0.01%
7 5% sucrose, 0.01% Silwet-L77	mGFP5-ER	0/3759 = 0		0/34,210 = 0

[a] contains modified *GFP* gene as selective marker.
[b] contains BAR^r (BASTA-resistance) and *GUS* gene as selective markers.
[c] contains BAR^r gene as selective marker.
[d] transformation efficiency: number of transgenic plants/ screened seedlings = % efficiency of transformation.

were used and the expected length of the amplified product is 418 bp. The amplification of the *GFP* gene fragment was done using the primers 5′-CATTTCATTTGGAGAGAACACG-3′ and 5′-GGTCTTGAAGTTGGCTTTG-3′ and the expected length of the amplified product is 625 bp. Amplification of both gene fragments involved 30 cycles of 94 °C (30 s), 54 °C (30 s) and 72 °C (1 min) in an Eppendorf Mastercycler.

3. Results

3.1. Generation and screening of transgenic *C. bursa-pastoris* plants

Three different plant lines (described in Section 2.1.) were tested for transformation. As organisms mediating transformation the *A. tumefaciens* strains GV3101 and LBA4404, and different kinds of inoculation media were tested for transformation efficiency. To find a convenient method for screening or selection of transformed plants, a modified *GFP* gene and the *BASTA-resistance* (BAR^r) gene were tested as marker genes. The BASTA-resistance was generated with two different transformation vectors.

In a first approach four different infiltration media (1–4 in Tables 1 and 2) were tested, both with either the *Agrobacterium* strain GV3101 (Table 1) or strain LBA4404 (Table 2) to check for the general transformation capability of the two different *Agrobacterium* strains. For this purpose plants from the lines 1947-wt and 1947-*Spe* were treated with both *Agrobacterium* strains; ripened seeds from 'dipped' plants were collected and screened for the expression of the marker genes *GFP* and BAR^r, respectively.

In a second transformation approach the plant line 1947-*Spe* was replaced by the line 1948-*Spe* since it became obvious that it was easier to synchronise flowering with the replacing line. In individual plants of 1947-*Spe,* time of bolting varied strongly, whereas bolting of 1948-*Spe* was much more synchronised. In this later approach only the *Agrobacterium* strain GV3101 was used, because in previous experiments it had shown better transformation efficiency (see below). To enhance this efficiency, promising inoculation medium 4 from the first transformations and differently composed new media 5, 6 and 7 (modifying medium 4) were tested (Tables 1 and 2).

Table 2
The effects of different compositions of infiltration media on the production of transformed seeds of plants dipped with the *Agrobacterium* strain LBA4404

Infiltration medium variants, according to Table 1	Vector used	Plant line	
		1947-wt	1947-*Spe*
1 3% sucrose, 0.05% Silwet-L77 1x MS-salts, pH 5.7	mGFP5-ER [a]	1/2581 = 0.04% [b]	0/9710 = 0
2 5% sucrose, 0.05% Silwet-L77	mGFP5-ER	0/8900 = 0	0/5195 = 0
3 5% sucrose, 0.005% Silwet-L77 1/2xMS-salts, 44 mM BAP, pH 5.7	mGFP5-ER	0/9900 = 0	0/2010 = 0
4 5% sucrose, 0.02% Silwet-L77	mGFP5-ER	2/8636 = 0.02%	0/3175 = 0

[a] contains modified *GFP* gene as selective marker.
[b] transformation efficiency: number of transgenic plants/ screened seedlings = % efficiency of transformation.

For selecting transformed T_1 plants, seeds from all floral-dipped plants were sown and five-day-old seedlings were examined for the expression of the appropriate marker gene. Plants that showed green fluorescence (Fig. 2a) were separated from the others and observed for two more weeks. Expression of the transgene could usually be observed over this time span, but after a few days, when the first leaves following the cotyledons arose, the intensity of the GFP signal became much weaker (Fig. 2b, c).

BASTA-resistant seedlings transformed with the plasmids pGPTV-bar and FGC5941cb1-3 which showed no signs of necrosis were separated from the remaining heavily affected ones (Fig. 2d), and further grown. To test plants of older age for expression of transgenes, 6 from a total of 36 plants transformed with pGPTV-bar were randomly chosen for a histochemical *GUS*-expression assay. Mature leaves of many of these plants showed at least partial staining. Blue staining was visible at the leaf disc around the vascular bundle and the tips of the leaf. Only occasionally the signal was relatively strong (Fig. 2e), but often almost as weak as in case of untransformed plants (Fig. 2f), so that the *GUS*-expression assay was not generally useful as a marker in our experiments and was therefore not longer used in our approach. Possible reasons for the failure of this usually reliable screening method are discussed below in Section 4.2.

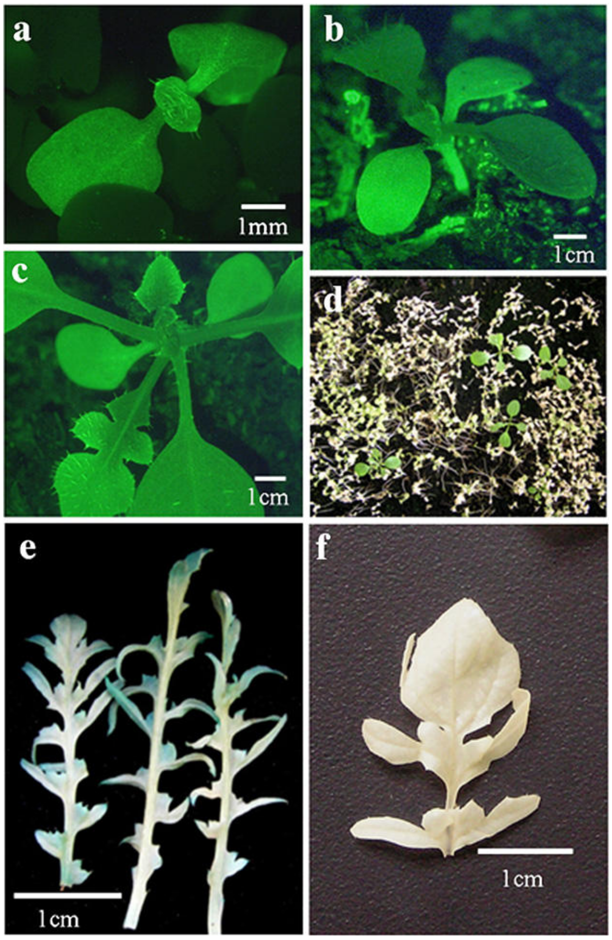

Fig. 2. Screening for selective markers in the T_1-progeny of the dipped T_0 plants; (a) 5-day-old green fluorescent seedling with one leaf partially covered by the leaf of an untransformed plant; (b) 8-day-old green fluorescent plant with diminishing fluorescence in the leaves following the cotyledons; (c) 14-day-old green fluorescent plant with stronger signal in the cotyledons and the marginal parts of mature leaves; (d) BASTA-resistant seedlings one week after treatment with BASTA; (e) GUS staining assay of randomly chosen leaves of a BASTA-resistant T_1 plant; (f) GUS staining assay with leaf of non-transformed plant.

3.2. Factors influencing transformation efficiency

For estimation of transformation efficiency seeds of the transformed T_0 plants were collected, counted and sown out for screening. The germination rates were determined and found to vary from 1% in few cases to mostly around 30–50% and up to 80% in rare cases. To compare transformation efficiency between the *Agrobacterium* strains GV3101 and LBA4404, all germinated seedlings of one experiment were counted against their number of positively screened transgenic plants and displayed in percentage of efficiency in Table 1 for GV3101 and Table 2 for LBA4404. Only the inoculation media 1 to 4 were included in that comparison. With the strain GV3101 we observed with medium variant 1 in one case a transformation efficiency of 0.28% and with medium variant 4 in several cases an efficiency of above 0.1%. In the experiments with LBA4404 the same media brought only two efficiency rates of 0.04% and 0.02% and in all other cases no transformants at all. These results suggest that *C. bursa-pastoris* shows different susceptibility towards different *Agrobacterium* strains.

For the strain GV3101 the influence on the transformation efficiency of the composition of the infiltration media used is summarised in Table 1. We mainly focused on analysing the influence of sucrose and Silwet L-77 on the efficiency to transform *C. bursa-pastoris*. Due to the cytotoxity of the surfactant Silwet L-77 different concentrations in the inoculation medium were tested. If a concentration of the surfactant of 0.02 or 0.05% (v/v) was used (medium 1, 2, 4, 5 and 6) transgenic plants could be generated at good rates of up to 0.4%. Decreasing the concentration of Silwet L-77 to 0.01% (medium 7) did not yield transformed plants. The concentration of sucrose in the inoculation medium was less critical. The use of 3, 5 or 10% (w/v) sucrose in the infiltration media resulted in comparable efficiencies in the production of transgenic *C. bursa-pastoris* plants. However, a concentration of 10% sucrose turned out to be most effective (media 5 and 6). The addition of MS-salts (media 1 and 3) did not boost transformation efficiency. In average, the media 5 and 6, containing 0.02 or 0.05% Silwet L-77, respectively, and 10% sucrose showed the highest transformation efficiency with *Agrobacterium* strain GV3101.

3.3. Molecular characterisation of transformed plants

All molecular characterisations have been performed with plants for which the *Agrobacterium* strain GV3101 had been used because of the poor efficiency of experiments done with the LBA4404-strain (compare Tables 1 and 2).

The presence of the *GFP* gene in plants transformed with mGFP5-ER was tested by PCR. DNA isolated from positively screened plants of the line 1947-wt was used as a template for a PCR with primers to the 5′-and 3′-end of the vector's *GFP*-

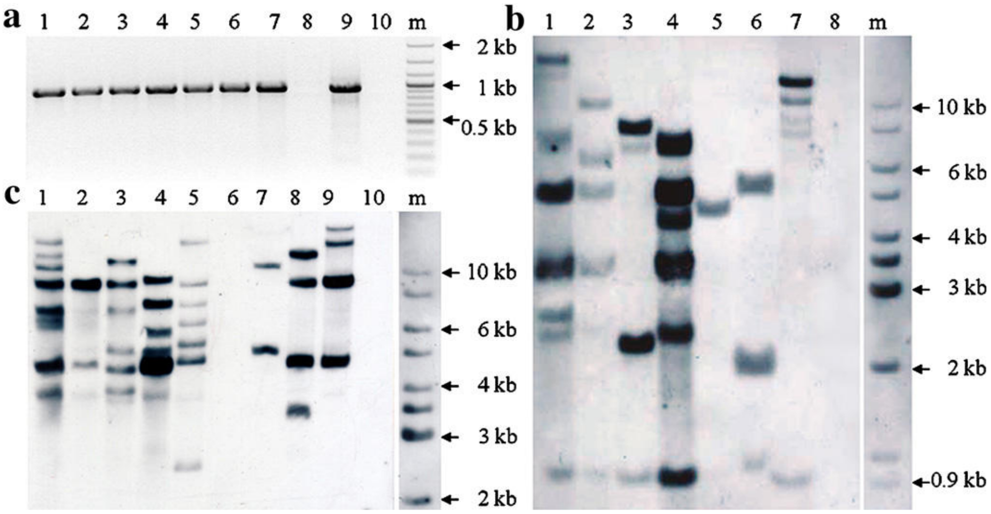

Fig. 3. PCR and Southern hybridisation experiments for verification of presence of transgenes in plant genomes; m: marker with length of some bands indicated (DNA Ladder Mix, Fermentas). (a) Products of a PCR with *mGFP*-primers on DNA extracted from plants transformed with mGFP5-ER; lanes 1-7: DNA of positively screened green fluorescent T_1-plants of plant line 1947-wt, lane 8: control DNA of non-dipped plant as template, lane 9: mGFP5-ER-Plasmid-DNA as template, lane10: negative control. (b) Southern hybridisation of genomic DNA obtained from plants transformed with mGFP5-ER; a DIG-labelled *mGFP* gene fragment was used as a probe; lanes 1–7: DNA of positively screened T_1-plants; the same DNA as in lanes 1–7 of (a) was used, lane 8: DNA of a 1947-wt (non-transformed) plant, the same as in (a). (c) Southern hybridisation of genomic DNA of plants transformed with pGPTV-bar; a DIG-labelled BAR^r gene fragment was used as a probe; lanes 1–5: DNA of BASTA-resistant T_1 plants and lane 6: control DNA of non-transformed plant, all of the plant line 1947-wt; lanes 7–9: BASTA-resistant T_1 plants and lane 10: DNA of non-transformed plants, all of the plant line 1947-*Spe*; kb; kilo bases.

sequence. In each case a band compatible with the expected size of 941 bp could be observed, whereas untransformed plant material showed no band at all (Fig. 3a).

To further test for a stable integration of the transgenes in the plant genomes Southern hybridisation was performed with the same T_1 plant DNA as in the former PCR test, with corresponding lane numbers in Fig. 3a and b. With this method the presence of the *GFP* gene in green fluorescent plants was confirmed. The putative number of copies of transgenes integrated into the genome could be estimated by the number of individual bands per lane in the DNA blot. Band numbers varied from one in lane 5 to eight bands in lane 1 in Fig. 3b, indicating insertion of transgenes at one to eight genomic loci. However, band intensities varied over a broad range, suggesting that in case of intensive bands multiple copies had been inserted. T_1 plants 1–4 and plants 5–6 respectively, all shown in Fig. 3b, arose from seeds collected from the same floral-dipped T_0 plant but exhibited a unique DNA band pattern in the DNA gel blots. Genomic DNA from non-transformed wild-type plants did not hybridise to the DIG-labelled *GFP* gene probe (Fig. 3b, lane 8). Also, the BAR^r-gene probe tested in a Southern hybridisation against the DNA isolated from T_1 BASTA-resistant plants of plant 1947-wt and 1947-*Spe* hybridised in a distinctive individual band pattern to the plant DNA (Fig. 3c). Here, the number of bands ranged from two in lanes 2 and 7 to eight in lanes 1 and 5 of the DNA blot when transformation with the vector pGPTV-bar was tested. Different band intensities were observed here as well. Even higher variation of band number from one to fourteen was observed in transformations where the vector pFGC5941cb1-3 had been used (data not shown). Here also unique band patterns occurred. In all molecularly tested transformation experiments single insertions were usually much rarer than multiple ones.

Table 3
Segregation of T_2 plants (offspring of selfed T_1 plants transformed with mGFP5-ER and pGPTV-bar)

Selfed T_1-plant [a]	Number of T-DNA-integrations	T_2-offspring		Segregation ratio	χ^2 (*P*-value)
		transgenic plants	non transgenic plants		
1947 *Spe* :: mGFP5-ER 2-1 A [b]	5	185	72	2.57 : 1	1.246 ($P>0.25$)
1947 wt :: mGFP5-ER 4-5	1	449	162	2.77 : 1	0.747 ($P>0.35$)
1947 wt :: mGFP5-ER 4-4	5	350	121	2.89 : 1	0.120 ($P>0.7$)
1947 wt :: mGFP5-ER 4-7	4	357	126	2.83 : 1	0.304 ($P>0.5$)
1947 wt :: mGFP5-ER 4-3	2	341	97	3.51 : 1	1.903 ($P>0.17$)
1947 wt :: mGFP5-ER 4-6	2	590	198	2.98 : 1	0.007 ($P>0.9$)
1947 wt :: pGPTV-bar 2-1 B [b]	1	220	60	3.66 : 1	1.905 ($P>0.15$)
1947 wt :: pGPTV-bar 2-2 C [b]	7	227	86	2.64 : 1	1.023 ($P>0.3$)

[a] all from population 1947 transformed with the *Agrobacterium tumefaciens* strain GV3101.
[b] in these plant generations Southern experiments were performed.

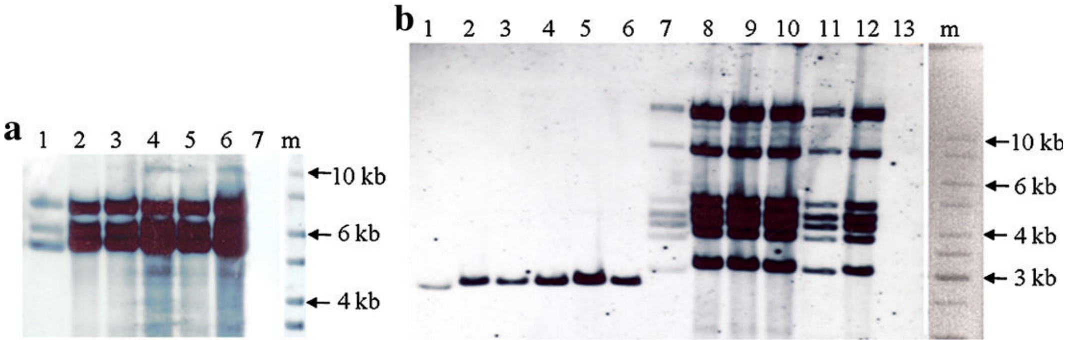

Fig. 4. Southern hybridisation of DNA from T_1 plants and offspring to detect stable inheritance of transgenes; DNA is less concentrated in T_1 parent plants due to different amounts of plant material available; m: marker with length of some bands indicated (DNA Ladder Mix, Fermentas). (a) Lane 1: band pattern of *T1 plant A*: 1947-*Spe* :: mGFP5-ER 2-1 (Table 3), lanes 2–6: DNA of five green fluorescent offspring T_2 plants; lane 7: non-transformed control plant 1947-*Spe*; a DIG-labelled *mGFP* gene fragment was used as a probe. (b) Lane 1: band pattern of *T1 plant B*: 1947-*wt* :: pGPTV-bar 2-1 (Table 3), lanes 2–6: DNA of five BASTA-resistant offspring T_2 individuals; lane 7: band pattern of *T1 plant C*: 1947-*wt* :: pGPTV-bar 2-2 (Table 3), lanes 8–12: DNA of five BASTA-resistant T_2-progeny individuals; lane 13: non-transformed *C. bursa-pastoris* DNA; a DIG-labelled *BAR* ^{r} gene fragment was used as a probe; kb: kilo bases.

To study the segregation pattern of the integrated T-DNA, six T_1 plants that were tested positive for the integration of the *GFP* gene and two BASTA-resistant plants were selfed. The resulting T_2 generations were analysed for marker gene expression.

The six T_2 generations which arose from the selfing of green fluorescent T_1 plants segregated in a ratio of approximately three fluorescent to one non-fluorescent plant. In all cases a segregation ratio close to 3:1 was supported by a *P*-value of above 0.17 (Table 3). A closer look at the expression of *GFP* in the individuals of the T_2 offspring uncovered differences in fluorescence levels at higher age (see above) even between siblings. A Southern experiment was done with the T_1 plant A (indicated in Table 3) and five randomly chosen transgenic members of its T_2 generation identified by green fluorescence. The same band number and pattern was confirmed in the T_1 plant and the tested transgenic offspring (Fig. 4a).

The approximate 3:1 segregation ratio was also confirmed when testing BASTA-resistant T_2 plants (Table 3). In the offspring generations of the two tested BASTA-resistant T_1 plants we found in one case a reliable *P*-value of above 0.3. In the other case we observed a *P*-value of above 0.15 (Table 3) both fitting to a 3:1 segregation model. Differences in expression level of the transgene have not been observed as with the fluorescent plants because the surviving plants appeared all equal after the BASTA treatment. Also with the GUS assay different expression levels were not observed due to the generally very weak staining signal. Southern blot analyses with parent and BASTA-resistant offspring of T_1 plant C (indicated in Table 3) revealed seven integrations in T_1 and offspring genomes. Experiments with T_1 plant B and respective offspring (indicated in Table 3) showed one integration in the T_1 parent plant and its progeny, all with the same band pattern (Fig. 4b).

4. Discussion

This is the first successful report on the production of transgenic *C. bursa-pastoris* plants. We demonstrate the expression of the introduced *GFP* and *BAR* ^{r} genes in seedlings and older plants, the integration of the foreign genes into the plant genome and a stable inheritance of the transgenes.

4.1. Effects of experimental conditions

For a successful transformation of *C. bursa-pastoris* the use of the appropriate *Agrobacterium* strain appeared to be essential. Acceptable transformation rates of above 0.1% were only obtained in the experiments with the strain GV3101 (Tables 1 and 2). Previously, Tague (2001) reported that *C. bursa-pastoris* could not be transformed in his experimental approaches with the strain LBA4404. This is consistent with the fact that also with *A. thaliana* the susceptibility for *Agrobacterium* transformation depends on the bacterial strains used (Clough and Bent, 1998; Bent, 2000). In line with this, a broad study with numerous *A. thaliana* ecotypes revealed a certain ability of some of them to resist an infection by *Agrobacterium* (Nam et al., 1997). Tague (2001) discussed a possible reason for his inability to transform *C. bursa-pastoris*. A low level of seed production is fortified in the process of flower submersion and vacuum infiltration, so that screening for positive transformants may have been too ineffective. In our investigation we encountered a related problem. The low amount of seeds in the range of 500–5000 from one T_0 *Capsella* plant compared to about 10 000–15 000 seeds from *A. thaliana* T_0 plants was worsened by very low germination rates in some cases (data not shown). Thus for successful screening for transformants one has to generate sufficient amounts of seeds by dipping more T_0 plants. Therefore, optimisation of germination rates, e.g. by gibberellin treatment, is a challenge of our future transformation experiments with *C. bursa-pastoris*. We tried to avoid the problem of sensitivity for submersion and vacuum infiltration by submerging our plants only for a few seconds with gentle agitation. This treatment seems to be more tolerable for *Capsella* flowers than dipping for several minutes under vacuum like in the experiments of Tague (2001). Despite the short immersion time we obtained good transformation rates. Similar observations have also been reported for *Arabidopsis* (Bent, 2000). We propose that using the alternative *A. tumefaciens* strain

GV3101 in concert with a more gentle dipping procedure was an appropriate way to overcome the former transformation inability of *C. bursa-pastoris*.

With the media variants 5 and 6, composed of 10% sucrose and 0.02 or 0.05% Silwet L-77, respectively, we could identify infiltration conditions to obtain transformation rates of approximately 0.1–0.4%. In parallel experiments performed with *A. thaliana*, where we used the same vectors and methods, we obtained average transformation rates of 0.4%–1.7% (data not shown). These values are similar to those reported in the literature, e.g. 0.6%–0.7% for *A. lasiocarpa* und 1%–2% for *A. thaliana* (Tague, 2001). The transformation efficiency in *C. bursa-pastoris* is thus lower, but still satisfactory in relation to the experimental effort and sufficient for a number of applications (see below).

4.2. Infection mechanism

Understanding the mechanism of transformation may help to set up protocols for other species to generate transgenic plants. In case of *A. thaliana* the ovule has been identified as the target of *A. tumefaciens in planta* transformation. Several studies revealed that the young ovule or the unfertilised egg is penetrated during the infection, causing all genetically altered plants arising from the same plant to be independent from one another (Ye et al., 1999; Desfeux et al., 2000; Bechtold et al., 2000).

The observation that even transgenic *C. bursa-pastoris* plants that arose from the same T_0 plant showed unique band patterns in the Southern blot analysis (Fig. 4a, b) suggests that the infection occurred at a developmental stage when the ovules have already been formed, thus indicating an infection mechanism similar to that of *A. thaliana*. Our results thus do not support an infection of meristem cells to be likely, in contrast to what has been suggested to be the case in generating transgenic radish (*R. sativus*) and *M. truncatula* plants (Curtis and Nam, 2001; Trieu et al., 2000).

The results of the DNA blot experiments revealed in all three tested cases no recombination in band patterns in the 5 chosen offspring plants (Fig. 4) and suggest, therefore, a block-wise integration of several T-DNA copies at the same locus in the genome. This might provide a clue concerning the mechanism which controls T-DNA integration into the *C. bursa-pastoris* genome, by suggesting that the access to a genomic region is the limiting step in these transformations. Even though the T_1 plant A and its offspring have the same number of insertions, indicated by band numbers in Fig. 4a, the GFP fluorescence levels showed a range from strong signals in young seedling stages but subsiding signals in older age, to continuously strong signals up to a few weeks of growth. This phenomenon was observed in other T_2 generations as well (C. B., data not shown). Nevertheless, low marker gene expression might have been caused by gene silencing effects, which may also apply to the GUS-staining signals of the BASTA-resistant plants in some of our transformation experiments. This silencing might occur whenever the number of inserted marker genes increases above a critical threshold. It will thus be a challenge of future experimentation to avoid multiple insertion of marker genes into the *Capsella* genome by changing some parameters, such as the *Agrobacterium* strains being used. The AGL-1 strain, which has a C58 chromosomal background just as GV3101 (Nam et al., 1997; Curtis and Nam, 2001), might result in transformed plants with fewer T-DNA insertions. In any case, the reasons why transgene expression levels decrease with plant age deserve further investigations.

4.3. Outlook

The possibility to transform *C. bursa-pastoris* opens the door for the development of several efficient genetic and genomic tools, such as the establishment of T-DNA-and transposon insertion-mutagenised populations that have been so successful in *A. thaliana* for investigation gene functions at a large scale (Alonso and Ecker, 2006). Even without establishment of mutagenised populations for *C. bursa-pastoris*, specific questions about gene functions could be answered. Here, a promising application would be the specific knock-down of genes of interest via RNA interference (RNAi) and the heterologous expression of genes in different model plants as mentioned before in the introductory part of this article (Baulcombe, 2004; Yoon and Baum, 2004).

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6 Discussion

6.1 Integrated discussion of the used methods and insights obtained from the corresponding results

6.1.1 Standard morphological and genetic methods answer fundamental questions about the nature of *Spe*

The first requirement in the establishment of a homeotic mutant of *C. bursa-pastoris* as a new model system was to analyse the phenotype of both available lines of the *Spe* variant carefully with morphological methods such as anatomical dissection, analysis of micro-structures with Scanning Electron Microscopy (SEM). Functional analyses such as staining tests for pollen viability and crossing experiments testing for fertility completed the analysis. This was conducted by a detailed genetic analysis (see manuscript II). SEM analysis of organ specific surface structures together with the functional analysis helped to classify the *Spe* variant as a homeotic mutant displaying a change of floral organ identity. SEM of young inflorescence apices demonstrated that the timing in which the organs in any whorl develop was not affected in the mutant compared to the wild-type flowers. In addition, SEM analysis resulted in an overview of developmental stages based on landmark events, similar to the staging analysis of *Arabidopsis* flower development conducted exemplarily by Smyth et al. (1990). All these detailed investigations of structure as well as of developmental stages are an important prerequisite for the reconstruction of developmental processes and, of course, for the description of other results such as *in situ*-hybridisation signals in sections of developing flowers. The micro-anatomical analysis reported in manuscript II is a fundamental information source not only whithin this thesis but also for future investigators dealing with floral development of *C. bursa-pastoris* and related Brassicaeae.

For the analysis of proper function of transformed stamens I used the method of pollen treatment with Alexander´s stain (Alexander 1969) to demonstrate viability of pollen cytoplasm via a positive staining result and with a crossing approach involving isolated pollen originating from second and third whorl stamens in *Spe* and wild-type plants. The number of seeds obtained from transformed stamens of the second whorl in comparison with positive and negative control experiments demonstrated, that pollen obtained from a transformed second whorl stamen is able to fertilise ovules of wild-type and *Spe* emasculated mother plants. Taken together, these functional tests did not only confirm the completeness of the homeotic conversion in the *Spe* variant, but also shed light on its evolutionary potential, as the additional functional stamens might contribute to pollination and consequentially to propagation in addition to the ´normal` third whorl stamens. This could provide a first step to new pollination procedures, e.g. wind pollination. The *Spe* phenotype could also be a starting point for differential or additional pollinator attraction, which would, however, not depend on full functionality.

The genetic analysis testing for the number of loci involved in the *Spe* phenotype was one of the most important initial experiments for the establishment of *Spe* as a model to study the impact of homeosis, as the crucial point of this study was to find a mutant in which a single change in a major locus occurred that results in a profound rearrangement of floral structure. My results obtained with classical genetic crossing experiments confirmed historical analyses (see Manuscript I; Dahlgren, 1919) of different decandric variants of *C. bursa-pastoris* suggesting, that in the cases analysed so far the *Spe* syndrome always results from to a single genetic change . In a modified test for allelism adopted according to the co-dominant inheritance of the phenotype, I demonstrated that the same or a very closely linked locus is responsible for the phenotype in both *Spe* lines that were investigated (for details of the modified test, see manuscript II). But as the expressivity of the *Spe* phenotype differed in both lines, I suggest either influences of a different genetic background or an independent origin of the mutations. The anatomical differences between the two analysed *Spe* lines and finally the fact, that a supposedly responsible deletion in the second intron of the *CbpAGa* gene in one of the *Spe*-lines is not detectable in the same region of the other *Spe* line (see below), corroborates the possibility of an independent origin of the mutation underlying the *Spe* phenotype. A co-dominant mode of inheritance was displayed by the intermediate F_1 phenotypes resembling chimeric organs where different gene products may compromise each other showing dose dependence. This was also observable by the 1:2:1 segregation pattern of F_2 generations and suggests the action of a given gene is more likely promoted rather than lost. Therefore, the co-dominant inheritance mode points to a mutation in a *cis*-regulatory element in a gene responsible for the shift of stamen identity in the *Spe* variant, representing a gain of function mutation (see also Figure 2 and manuscript I). Quite some examples involving transcriptional regulatory genes where co-dominance goes along with mutations in *cis*-regulatory regions exist in plants, including *Knotted1*, *Gnarley1* and *Rough Sheath1* (maize), *Hooded* (barley) and *Ovulata* (*Antirrhinum*) (Vollbrecht et al., 1991; Foster et al., 1995; Schneeberger et al., 1995; Müller et al., 1995; Bradley et al., 1993). Of special interest in conjunction with the *Spe* syndrome is the

Ovulata mutant of *Antirrhinum*. Here, the phenotype resembles in part that of *Spe* with (apetals replaced by stamens and staminoids mong other pleiotrophic effects such as transformation of sepals into carpels). Responsible for the *ovulata* phenotype is the insertion of a Tam3 transposon in the second, *cis*-regulatory region of the class C gene *PLENA* (*PLE*), causing ectopic expression of that gene in the outer two whorls in addition to ectopic expression in other floral organs and vegetative tissues of *Antirrhinum* with the corresponding phenotypical consequences (Bradley et al., 1993). Very likely, the transposon integration led to the destruction of a regulatory binding site of a transcriptional regulator of *PLE* that is normally repressing transcription of that gene in all tissues, except of the two inner floral organ whorls. A related mechanism was also hypothesised for the *Spe* variant in manuscript I, but the change in transcriptional regulation was assumed to take place only in the second floral whorl, as only the corresponding organs types are affected in *Spe*.

6.1.2 Isolation of *AG*-like gene orthologs from *C. bursa-pastoris*

The aim of the molecular analysis of the *Spe* variety was to verify wether a candidate class C gene ortholog shows ectopic expression as hypothesised above and consequently to elucidate the molecular mechanism causing the change in expression pattern in a candidate gene approach (Manuscript I). Initially, I isolated the cDNA fragments of orthologues of floral organ identiy genes of *Arabidopsis* to study their expression in wild-type and *Spe* flowers by the RACE (Rapid Amplification of C-DNA Ends)- cloning to obtain sequence information for expression analysis (probe construction) and for anchor exon-fragments that facilitate isolation of the genomic gene sequences. In all cases I isolated two co-orthologues of the organ identity genes *AGAMOUS* (*AG*), *APETALA1* (*AP1*), *APETALA3* (*AP3*), and *PISTILLATA* (*PI*), as expected considering the tetraploid genome of *C. bursa-pastoris*. The second class A organ identity gene *APETALA2* (*AP2*) known from *Arabidopsis* was excluded from the analysis, because it is reported to be expressed in all floral (and vegetative) tissues and regulated post-transcriptionally (Jofuku et al., 1994). In addition to the *AG* co-orthologues the other members of the *AG*-like clade, *SHATTERPROOF1* and *2* (*SHP1, 2*) and *SEEDSTICK* (*STK*) were isolated. The founding gene of that clade was the *AG* gene of *Arabidopsis* providing C-function (Becker and Theißen, 2003). All members of this clade, except *STK*, have been shown previously to confer stamen identity to second whorl organs when expressed ectopically in the outer floral whorls and were therefore also considered as candidates for ectopic expression in the second floral whorls (Pinyopich, 2003; Favaro et al., 2003). I performed Southern hybridisation of wild-type and *Spe* genomic DNA fragments with genomic probe fragments of the *AG*-like orthologues. This was done in order to clarify, wether any obvious duplication of these orthologues occurred besides the duplication that led to the origin of tetraploidy in *C. bursa-pastoris*. The probe fragments were isolated with the help of cDNA fragments provided by Janine Ziermann. Band patterns in these experiments only showed four co-orthologues bands in the case of the *CbpSHP1*. This could either represent additional duplicates or allelic polymorphisms. A definitive exclusion of any of these alternatives is not possible yet.

The correct classification of the isolated genes was checked in a phylogenetic reconstruction involving *AG*-like proteins from other angiosperm groups. The resulting phylogenetic tree was rooted with the *AG*-orthologue of a gymnosperm species, *Gnetum gnemon*. We used the distance based method of Neighbour Joining for phylogeny re-construction and tested the reliability of the trees with bootstrapping (Saitou and Nei, 1987; Felsenstein, 1985). Neighbour Joining proved to be effective previously for analyses of MIKC-type MADS domain protein relationships (Theißen et al., 2000; Becker and Theißen, 2003).

The cDNA fragments were also the basis for construction of RNA-probes used in the *in situ*-hybridisation experiments via in vitro transcription.

6.1.3 Comparative analysis to demonstrate ectopic expression of C-function genes in *Spe*

I choose the *in situ*-hybridisation method for expression analysis of organ identity genes, because it already detects the onset of transcription of these genes in very early stages of floral development and in addition, the exact spatial distribution of expression signals in the tiny floral organ primordia. Other standard methods of expression analysis, like Northern Hybridisation or quantitative Real-time PCR are less useful due to practical reasons con-cerning the collection of very young floral organ tissues. Another possibility of expression analysis would be a reporter gene approach like the fusion of the promoter of a gene of interest with a reporter gene such as the *GREEN FLUORESCENT PROTEIN* (*GFP*) or β-glucuronidase (*GUS*). However, the isolation of the complete specific and functional pro-moters and other regulative regions like the second intron of the AG-like genes for all floral identity genes, was regarded as inadequate at this stage of analysis of the *Spe* phenomenon for reasons of efficiency, as it seemed more effective to first confine the search for a ´hot candidate` gene. Therefore, these kinds of analyses are not in the scope of this thesis, but further promoter analyses will be on the agenda of future works with *C. bursa-pastoris* to accomplish transgenic complementation analysis with a candidate mutant allele causing the *Spe* syndrome (see below).

The protocol for *in situ*-hybridisation was adapted from the protocol reported by Zachgo (2002) with the help of positive control probes, such as a marker for replicating cells, the *Histone 4* (*H4*) gene, that shows pattern of single stained cells scattered over growing tissues and the *AP3* orthologue that was expected to be expressed only in the second and third floral whorl in early stages of development like in *Arabidopsis* (Brandstädter et al., 1994; Groot et al., 2005; Jack et al.,1992) The positive as well as the negative controls with sense RNA probes of the investigated genes helped to find adequate experimental conditions to distinguish background staining signals from specific hybridisation signals. For probe generation, 3´UTR sequences were included to maximise the length of specific probes whereas the conserved MADS-box sequence element was excluded to avoid cross hybridisation with other MADS-box family members. Especially the probes of *AG*-like genes were checked for satisfactory sequence diversity using sequence alignments. We did not differentiate between the co-orthologues of the genes, as their sequences showed only minor differences to each other that would not suffice to resolve distinct hybridisation patterns.

In summary, throughout all *in situ*-hybridisation experiments only two *AG*-like genes showed ectopic expression patterns in the *Spe* flowers as compared to wild-type flowers. I demonstrated that *CbpSTK* is differentially expressed in wild-type and *Spe* flowers, in that *CbpSTK* is also detectable in the developing second whorl stamens as well as in the inner stamen and carpel whorls, while wild-type flowers of the respective stages display no signal at all. Interestingly, early *CbpSTK* expression at stages when primordia of the third (stamens) and fourth (carpel) whorl arise is not known so far from the corresponding orthologues of *Arabidopsis* or *Antirrhinum* (Rounsley et al., 1995; Colombo et al., 1995). The *CbpAG* transcription signal in stamens and carpels in the inner whorls of wild-type flowers followed exactly the expected pattern, whereas additional ectopic expression of *CbpAG* was detected in the second floral whorl of *Spe* flowers until late developmental stages. Taken together, expression signals of *CbpAG* and *CbpSTK* accompanied the organ identity shift in the second floral whorl. However, assuming that both of them have the potential to confer stamen identity to second whorl organs, it is still not possible to decide whether we observe a case of redundancy of direct regulation (parallel mis-regulation of redundantly acting genes by a mutated trans-acting factor) or if one gene influences organ identity indirectly by regulating the other. Such a question was very likely answered by the co-segregation of exclusively the *CbpAGa* gene with the *Spe* phenotype, suggesting that *CbpSTK* expression is positively regulated by the *CbpAGa* gene. The ectopic expression of *CbpAGa* might therefore be the initial responsible switch in the *Spe* flowers that directly determines stamen organ identity in the second floral whorl, as hypothesised in the modified ABC model shown in Figure 3. Guided ectopic expression exclusively in the second floral whorl of *Arabidopsis* through the class B gene promoter of *AP3* has previously been demonstrated to produce exactly the same specific organ transformation (Jack et al., 1997). Together with the already mentioned staminoidy in second whorl organs upon ectopic expression of *AG*, *SHP1* and *SHP2* (Pinyopich, 2003), this specifity of organ identity shift strongly corroborates our suggested modified model for *Spe* floral organ determination (Figure 3).

The analysis of floral identity genes in *C. bursa-pastoris* also gave insights into the conservation of expression profiles and corresponding functions of the other floral organ identity genes in Brassicaceae. The B function genes *CbpAP3* and *CbpPI* in *C. bursa-pastoris* wild-type and *Spe* plants showed exactly the same expression pattern as in *Arabidopsis*, suggesting that they provide also the same function (Jack et al., 1992; Goto and Meyerowitz, 1994) (Figure 3). Expression of *CbpAP1*, attributed to class A function in *Arabidopsis*, shows the same early expression in flower primordia and in the first developmental stage, when organ primordia of sepals arise in *Arabidopsis* and *C. bursa-pastoris*, suggesting similar function in determination of floral meristem identity. However, expression of *CbpAP1* was not detectable in the following stages in *C. bursa-pastoris* in contrast to enduring expression of *AP1* in *Arabidopsis* up to late developmental stages (Mandel et al., 1992; Gustafson-Brown et al., 1994). In addition, *CbpAP1* expression is terminated before the onset of *CbpAG* and *CbpSTK*. These expression patterns propose a less developed or lost function conserved function in organ identity determination and lack of mutual spatial repression in *C. bursa-pastoris*, as displayed in the modified ABC models in Figure 3. Alternatively, the (restricted) *AP1* function of *C. bursa-pastoris* represents an ancestral state and function as organ identity gene was fortified in *Arabidopsis*.

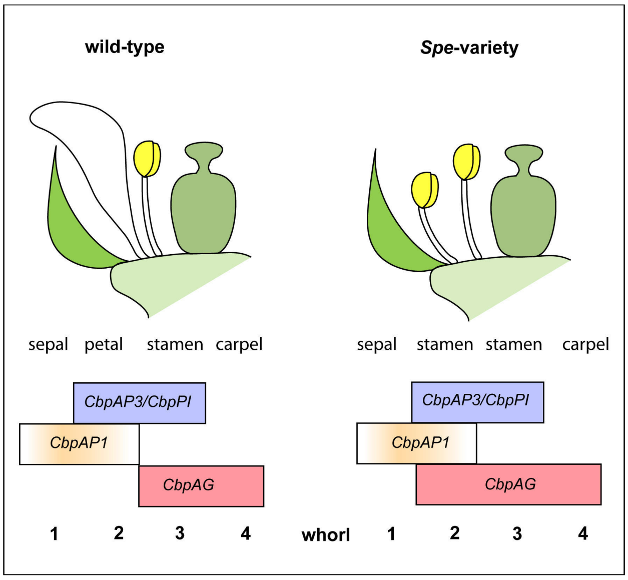

Figure 3. Modified ABC models for the regulation of floral development in *C. bursa-pastoris* wild-type and *Spe* plants.

6.1.4 Co-segregation analysis of candidate loci localise the gene mutated in *Spe*

A candidate gene approach was designed to clarify, whether one of the *AG*-like genes that showed ectopic expression in the second whorl is itself responsible for this expression patterns by changes in *cis*-regulatory regions, or whether a trans-acting gene induces ectopic expression signals. Considering the results of the genetic analysis, namely the co-dominant inheritance mode of *Spe*, that suggested a gain of function in the gene responsible for the *Spe* phenotype, we focused the analyses to the *AG*-like genes. In the experimental approach, conducted by Janine Ziermann in the frame of her PhD work, eight *AG*-like co-orthologues were analysed for Single Nucleotide Polymorphisms (SNPs) to establish markers distinguishing between wild-type and *Spe* genomic sequences of parent plants of a wild-type x *Spe* cross. One SNP of each co-orthologue was tested for co-segregation with the *Spe* phenotype, using the pyrosequencing method (Groth et al., 2006). Only the SNPs of the *CbpAGa* co-orthologue showed perfect co-segregation with the phenotypes of 191 F_2 offspring plants, whereas the seven other *AG*-like genes did not. Therefore, the *Spe* locus is either identical to the *CbpAGa* locus or at least very closely linked to it. Consequentially, the non-coding regions corresponding to regulative regions known from *Arabidopsis* as well as the non-coding sequences upstream of the 5´UTR were intensively screened for candidate sequence changes between wild-type and *Spe* parent plants of the mapped population. Only one remarkable sequence polymorphism was identified, a deletion of 22 bp combined with a substitution of 3 bp in the second intron, a region known to be the main regulative site in *Arabidopsis AG* (Sieburth and Meyerowitz, 1997; Bush et al., 1999; Deyholos and Sieburth, 2000, Hong et al., 2003). A number of positively and negatively acting regulators causing stamenoid petals upon mutation are known for *Arabidopsis*, most of which disqualify themselves either through pleiotropic phenotypic effects or through binding sites that do not fit to our candidate polymorphism, as discussed in detail in manuscript II. Only the orthologue of the *RABBIT EARS* (*RBE*) remains as candidate negative regulator of which the binding site in *CbpAGa* might be destroyed in the *Spe* allele. *RBE* encodes a Zn-finger protein which contains an AGT-core sequence as binding motif (Krizek et al. 2006), a motif that is apparent in the polymorphic site. But still, the *rbe* loss-of function phenotype is much weaker than the *Spe* phenotype, i.e. more than the binding site of just one regulative gene could be altered in the *Spe* allele. A closely linked factor regulating *AG* expression cannot be excluded as being responsible for the ectopic expression so far. Therefore, the logical next step for future experiments will be the transformation of the full length *CbpAGa* genomic sequence originating from a *Spe* plant line into a wild-type plant to test, whether the resulting phenotype resembles the *Spe* syndrome. If this turns out to be the case, the involved regulators and binding properties have to be investigated for both of the *Spe* lines analysed in this thesis, e.g. with protein-DNA binding studies.

6.1.5 Germline transformation of *Capsella bursa-pastoris* facilitates further functional studies

In order to provide a facility for transgenic functional analysis in *C. bursa-pastoris*, germline transformation via the ´floral dip` method was established in the diploma thesis of Conny Bartholmes and published afterwards (Manuscript III). As a first step, transformation was conducted with two different *Agrobacterium* strains and standard experimental conditions, as already reported as appropriate for other Brassicacae ´floral dip` protocols (Clough and Bent, 1998; Curtis and Nam, 2001; Tague, 2001). In this step the general capability of *C. bursa-pastoris* to be transformed was shown using either the modified *GREEN FLUORESCENCE PROTEIN* (*GFP*) as marker gene, or the *BASTA-resistance* (*BAR* ^{r}) gene and/or the *β*-glucuronidase (*GUS*) gene. Molecular verification of transgene integration in the genome was carried out by PCR amplification of partial fragments of marker genes and Southern hybridisation with probes containing marker fragments both of DNA of plants positively screened for marker expression and of non transformed negative control plants. Also the stable inheritance of the integrated transgenes in the genome was tested by selfing of T1 plants and analysis for segregation in the T2 generation as well as Southern hybridisation of T1 plant DNA in comparison to DNA of T2 plants expressing the respective marker. In those experiments, beside confirmation of stable inheritance the approximate segregation ratio of 3:1 also points to aof integration of transgenes into the genome happened blockwise with all the transgenes closely linked together on a single chromosome.

After demonstration of the general susceptibility for transformation of *C. bursa-pastoris*, the transformation rate was optimised using the *Agrobacterium* strain GV3101 and variable compositions of infiltration media. Tague (2001) reported previously, that in his experimental approaches with the *Agrobacterium* strain LBA4404 *C. bursa-pastoris* could not be transformed. In our experiments transformation efficiency with that strain was very poor as well. This is not surprising as the susceptibility of *Arabidopsis* for *Agrobacterium* transformation is dependent on the bacterial strains used as well and sometimes even completely disabled in several *Arabidopsis* ecotypes (Clough and Bent, 1998; Nam et al., 1997). Such a restriction may hold also for the closely related *C. bursa-pastoris*. With the use of the more appropriate *Agrobacterium* strain GV3101 and more gentle treatment of submersed plants without vacuum infiltration, a more tolerable treatment for *Capsella* flowers, we were able to overcome those obstacles. With the two most effective media variants we found lower but still sufficient transformation rates of 0.1-0.4% in *Capsella* in comparison to the 0.6-0.7% and 1-2% reported for *A. lasiocarpa* and *Arabidopsis*, respectively.

However, a phenomenon of fading *GFP* expression with growing age of transformed seedlings suggests, that gene silencing effects play a role when multiple transgenes are integrated in the tetraploid genome of *C. bursa-pastoris*. Future investigations with additional *Agrobacterium* strains known for lower insertion numbers, such as the AGL-1 strain (Nam et al., 1997) might demonstrate whether less transgene integrations results in lower gene silencing events.

6.2 The *Spe* variety represents a very special type of homeosis

In contrast to many well investigated floral homeotic mutants, the *Spe* variety of *C. bursa-pastoris* is fully fertile and seems to propagate efficiently enough to compete with wild-type plants in natural habitats (Manuscript I and Hintz et al., 2006). Why does it have a chance in nature at all? The answer to this question lies in the special character of the *Spe* phenotype which is replacement of sterile perianth organs by functional reproductive organs in general and in the quality the homeotic change of only one affected organ type without obvious pleiotropic effects. Specifically, the fertility of the regular sex organs in the 3rd and 4th whorl is not affected in *Spe* flowers, and even the additional stamens in the

2^{nd} whorl are fertile. Replacement of sterile organs by additional reproductive organs in self-fertile plant species is much less deleterious, given that propagation in an affected plant, namely fertilisation, does not depend on pollinator attraction, like in the predominantly selfing species *C. bursa-pastoris*. An additional point making the *Spe* phenomenon quite peculiar is that it occurs in a supposedly allopolyploid species, which is compensating very low outcrossing rates of about 0-20% by an effect called fixed heterozygosity (Hurka et al., 1989; Hurka and Neuffer, 1997; manuscript I). This effect may help to avoid imbreeding depression so that low outcrossing rates may be of minor importance in *C. bursa-pastoris*. Interestingly, preliminary results of a field study in which insect visitation of wild-type and *Spe* plants were compared under natural conditions, showed that *Spe* plants do not suffer from a complete lack of visitation of potentially pollinating insects (Manuscript I, preliminary data of J. Ziermann).

Taken together, in the *Spe* variant of *C. bursa-pastoris* many aspects joined together in a fortunate way allowing a remarkable structural diversification without obvious loss of plant fitness.

6.3 Implications for flower development and evolution in other model systems

As described above and in manuscripts I and II, the syndrome of petals transformed into stamen or stamenoid organs is not abundant, but also not extremely rare (Murbeck, 1918; Ronse De Craene, 2003). With this fact in mind it is surprising that no decandric variant is apparent in *Arabidopsis* and one could suggest that either a similar mutation has by chance not occurred so far, or, hypothesising even further, this kind of homeotic change is only possible in *Arabidopsis* through more than one mutational event. Therefore, stable transformation with the full length genomic fragment of the *CbpAGa* gene with the allele carrying the candidate polymorphism will also deepen our understanding of *Arabidopsis* floral development. The analysis of exact mechanism of the regulative switch in the *CbpAGa* gene in *C. bursa-pastoris* will inevitably shed light also on processes of C-functional gene regulation which could be of more general importance in flower development, such as characterisation of regulative binding sites of floral organ identity genes.

6.4. Conclusions and outlook

Considering all pieces of evidence, the results of my morphological, genetic and molecular gene expression analyses demonstrate, that the *Spe* phenotype represents a co-dominant mutant, wich causes perfect homeotic shifts in the organ types and positions which are tolerable for a plant competing within the limits of natural populations. The results also suggest, that ectopic expression of *CbpAG* is directly responsible for transformation of petals into stamens in the second floral whorl of the *Spe* phenotype. In addition, a candidate mutation was identified in the *cis*-regulatory region of the *CbpAGa* gene, a locus that is co-segregating with the *Spe* phenotype (work of J. Ziermann).

In evolutionary terms the *Spe* model demonstrates the potential of floral homeotic genes to induce novel flower structures by neo-functionalisation facilitated by a *cis*-regulatory modification of only one organ identity gene. In other words, the hierarchical position of organ identity genes within the regulating gene network enables perfect homeotic changes that have the potential to generate novel floral structures with only one mutational switch. Taken together, this thesis established the *Spe* mutant of *C. bursa-pastoris* as a model system to investigate the homeotic change from petals into stamens in particular and the species *C. bursa-pastoris* in general as an additional valuable model system to study development and evolution of flowering plants.

Regarding the future of the *Spe* model, the most obvious future experiments will be the verification of the candidate mutation in the *cis*-regulatory region of *CbpAGa* via transgenic modification experiments of *Arabidopsis* and *C. bursa-pastoris*, a method that was published for the latter species in the frame of this thesis. During these experiments full length genomic sequences of the *CbpAGa* allele from both *Spe* lines should be transformed to *Arabidopsis* and wild-type *C. bursa-pastoris* plants to test if the same dominant phenol-type arises upon transformation. The candidate mutation in the second intron of one *Spe* line has to be verified and, as the other *Spe* line was shown to be allelic but not mutated at the same region, the *CbpAGa* locus has to be screened for a candidate polymorphism in this other line.

For the evaluation of the evolutionary potential of the *Spe* variant, fitness parameters have been tested under natural environmental conditions, such as pollinator visitation, fruit and seed production and germination rates in the frame of another PhD work (J. Ziermann). After exact molecular verification of the mutational event leading to *Spe*, the mutated sequence element will be of much help to test whether the *Spe* locus is under purifying or positive selection in the natural populations of both of the variants.

7. Summary

In the evolution of floral structures a number of examples exist where homeotic changes in flower organisation probably occurred at the base of taxonomic groups. It is, however, not known how exactly natural morphological diversity is generated, especially how homeotic changes which originated spontaneously in the wild get fixed in a population. A plant model system in which the role of a spontaneous homeotic mutation can be studied in a multidisciplinary approach combining morphological, genetic, molecular and ecological methods was not available so far. In this thesis I established the natural floral homeotic *Spe* (*Stamenoid petals*) variety of *Capsella bursa-pastoris*, in which petals are completely transformed into stamens, as such a model system.

In a concomitant review article known facts about the role of homeosis were compiled to demonstrate the potential of *C. bursa-pastoris* as a model species and for introduction of the *Spe* variant. A hypothesis on the underlying molecular mechanism based on ectopic expression of a class C organ identity gene as well as an experimental program to study this phenomenon with ecological and evolutionary methods was developed in the review article.

With my experimental results I demonstrated that the *Spe* variety shows a complete homeotic shift from petals to stamens that is driven by an allele of a single co-dominant locus in two different *Spe* variety lines without pleiotropic effects. The co-dominant mode of inheritance of the *Spe* phenotype suggested a gain-of-function mutation in a regulatory region of the same gene in two independent *Spe* variety lines. I also showed that ectopic expression of two *AG*-like genes, *CbpAG* and *CbpSTK*, correlate with organ transformation, of which very likely the *CbpAG* ectopic expression is directly responsible for the changed organ identity. Results of parallel PhD work of J. Ziermann demonstrated that exclusively the class C gene *CbpAGa* co-segregates with the mutant phenotype and that likely a change in a candidate *cis*-regulatory region of the *CbpAGa* gene is responsible for the deviating expression patterns of this gene. These results have been included into the publication of my analyses.

Finally a protocol for stable germline transformation of *C. bursa-pastoris* by the ´floral dip`method established by C. Bartholmes during her diploma thesis under my supervision, was published as a tool for functional analysis in *C. bursa-pastoris*.

Taking together, the *Spe* model system promoted essential experimental approaches such as studies of the underlying molecular mechanisms of mutant origin and establishment of new tools for functional mutant analyses to encourage further analysis of *C. bursa-pastoris* in the frame of evolutionary developmental biology.

Zusammenfassung

Es existieren einige Beispiele in der Evolution floraler Strukturen, bei denen vermutlich homöotische Veränderungen an der Basis taxonomischer Gruppen stattfanden. Es ist jedoch noch nicht bekannt, wie morphologische Diversität generiert wird und zwar speziell wie spontan auftretende homöotischen Veränderungen in einer natürlichen Population fixiert werden. Eine Modellpflanze, in der spontane homöotische Mutationen in einem multidisziplinären Ansatz studiert werden konnten, war bis jetzt nicht verfügbar. In meiner Arbeit habe ich die natürlich auftretende florale homöotische Variante *Spe* (*Stamenoid petals*) in *Capsella bursa-pastoris* als neue Modellpflanze etabliert. In dieser Variante sind die Petalen des zweiten Blütenkreises durch Stamina ersetzt.

In einem begleitenden Übersichtsartikel wurde das Potential von *C. bursa-pastoris* als Modellsystem dargestellt und die *Spe*-Variante als Studienobjekt eingeführt. Eine Hypothese zum molekularen Ursprung des *Spe*-Phänotyps wurde vorgestellt, der zufolge die Organtransformation im zweiten Blütenkreis auf die ektopische Expression eines Gens der Klasse C zurückgeht und ein Programm zu detaillierten Feldstudien, die methodische Ansätze aus Ökologie und Evolutionsbiologie vereinen, vorgestellt.

Die Ergebnisse meiner Experimente zeigen, dass die *Spe*-Variante auf eine vollständige homöotische Umwandlung von Petalen in Stamina zurückgeht. Diese Umwandlung wird in zwei unabhängigen mutanten Linien ohne pleiotrope Effekte durch den gleichen co-dominant vererbten Lokus verursacht. Schon die co-dominante Vererbung legt nahe, dass es sich bei der *Spe*-Variante um eine „gain-of function“ Mutation handelt.

Ich habe ebenso gezeigt, dass die ektopische Expression von zwei Genen, *CbpAG* und *CbpSTK*, mit der Organtransformation korreliert. Sehr wahrscheinlich ist jedoch nur die ektopische Expression von *CbpAG* verantwortlich für die veränderte Organidentität. Die Ergebnisse einer weiteren Promotions-Arbeit von J. Ziermann stützen diese Aussage durch die Co-Segregation von Kandidatengenen mit dem mutanten Phänotyp, die einzig für den Lokus von *CbpAGa* gezeigt wurde. Die abweichende Expression ist wahrscheinlich auf Veränderungen in der *cis*-regulatorischen Region des *CbpAGa* Gens selbst zurückzuführen. Diese Ergebnisse flossen in die Publikation meiner oben genannten Ergebnisse mit ein.

Schließlich wurde im Zuge der von mir betreuten Diplomarbeit von C. Bartholmes ein Protokoll zur stabilen Transformation von *C. bursa-pastoris* mittels der ´floral dip`-Methode etabliert und publiziert.

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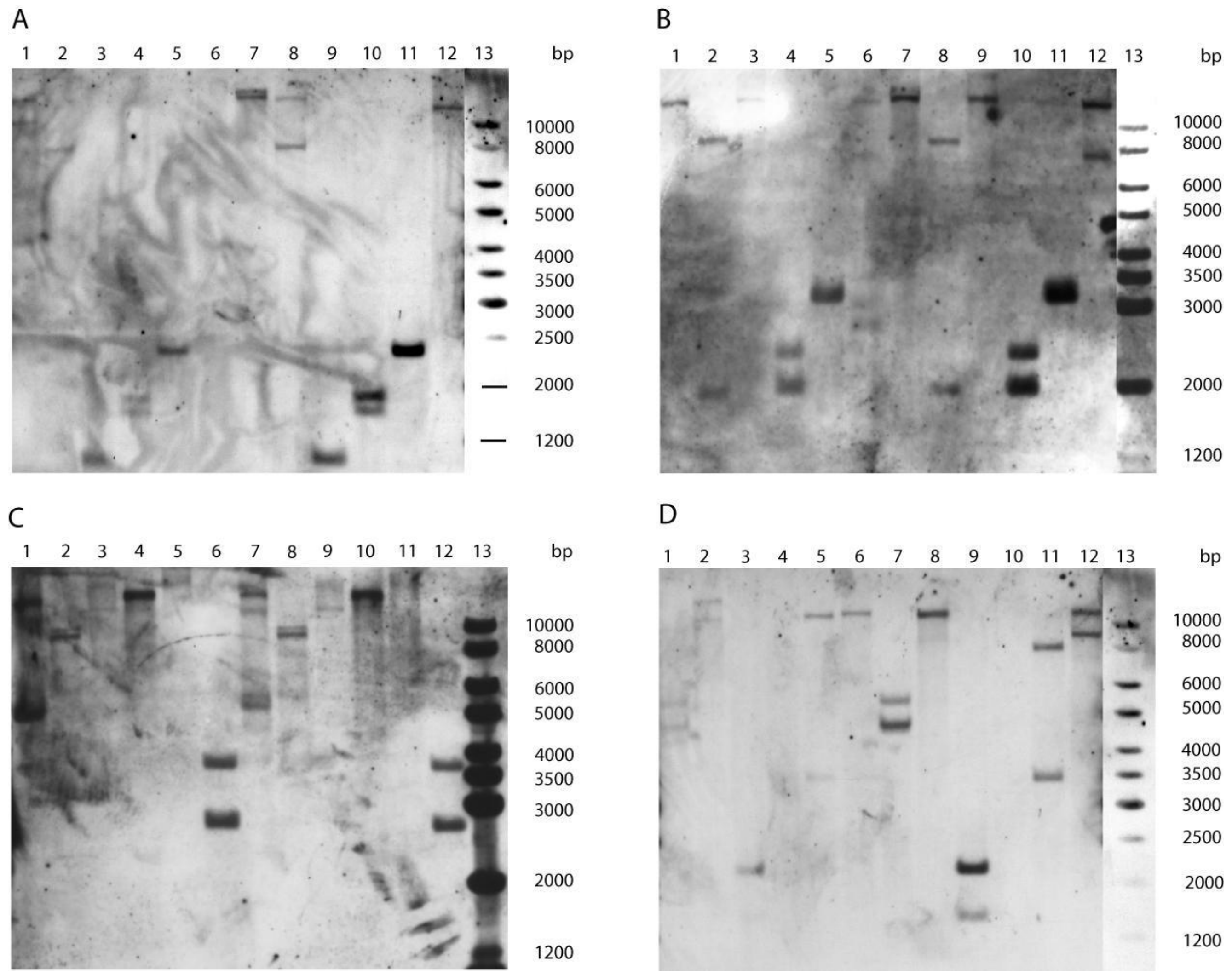


Supplemental Figure 1.

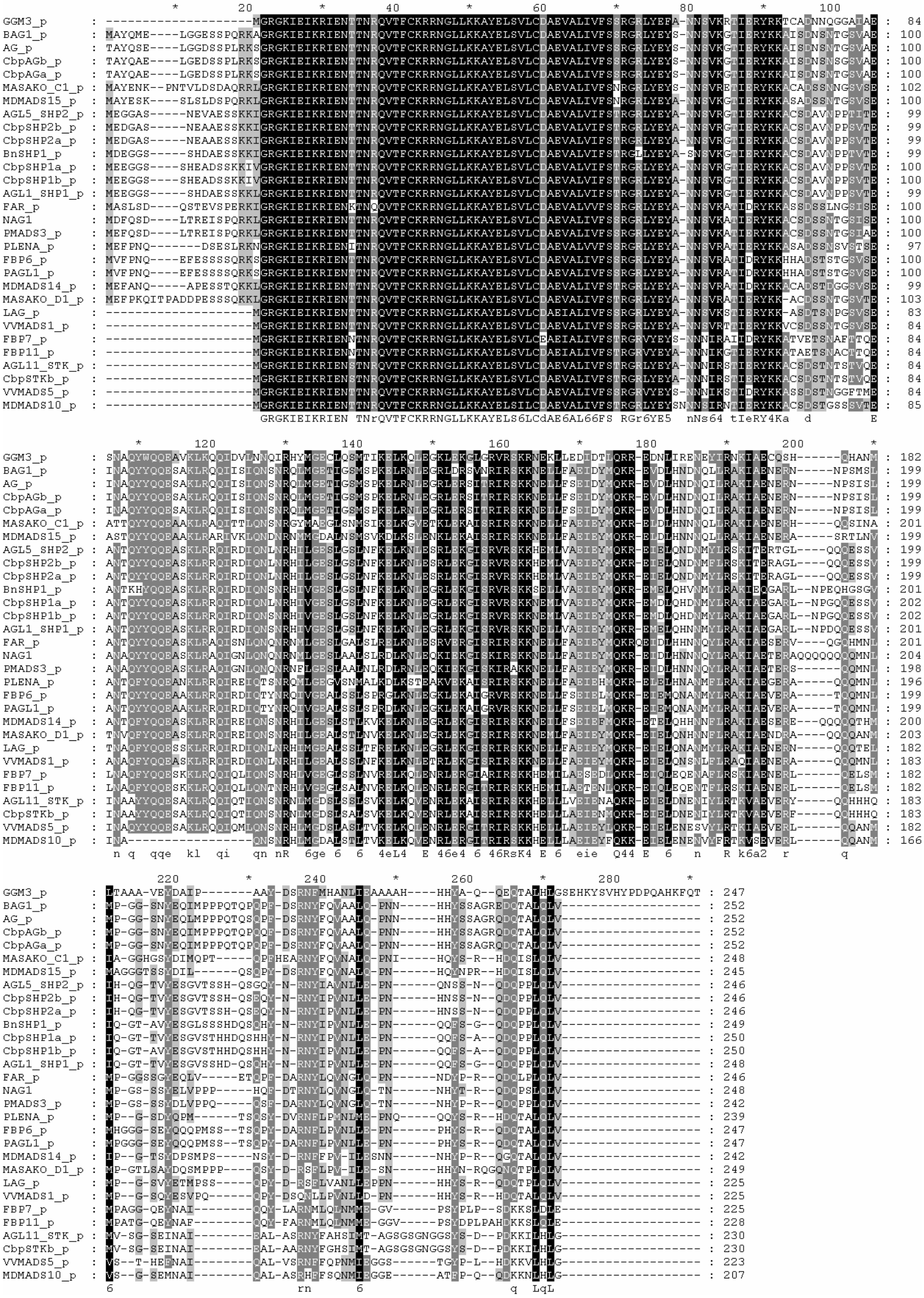
DNA blot hybridization experiments with genomic DNA (30µg) of 1947-wt and 1947-*Spe* plant leaf material digested with six different restriction enzymes, separated on 1% (w/v) agarose gel and blotted onto positively charged nylon membranes. Membranes were hybridised with DIG- labelled gene fragments according to manufacturer´s instructions. Hybridised fragments comprise genomic sequence from beginning of I-domain to the C-domain spanning four to five introns of the respective genes.
Lanes (1)-(6) genomic DNA of 1947-wt plants; lanes (7)-(12) genomic DNA of 1947-*Spe* plants.

(A) Hybridization with *CbpAG* fragment. DNA digested with restriction enzymes (1), (7) EcoRI; (2), (8) EcoRV; (3), (9) DraI. (4); (10) HincII *(cuts the probe once in K-domain)*; (5), (11) XbaI; (6), (12) XhoI.

(B) Hybridization with *CbpSTK* fragment. DNA digested with restriction enzymes (1), (7) EcoRI; (2). (8) EcoRV; (3), (9) DraII; (4), (10) HincII *(cuts the probe in intron between K1 and K2 domain)*; (5), (11)XbaI; (6), (12) XhoI.

(C) Hybridization with *CbpSHP1* fragment. DNA digested with restriction enzymes (1), (7) EcoRI; (2), (8) EcoRV (3), (9) SalI; (4), (10) XbaI; (5), (11) XhoI; (6), (12) HincII *(cuts the probe right at the 5´end of fragment)*.

(D) Hybridization with *CbpSHP2* fragment. DNA digested with restriction enzymes (1), (7) EcoRI *(cuts the probe once in intron between K1 and K2 domain)*; (2), (8) EcoRV; (3), (9) DraI; (4), (10) HincII *(cuts the probe three times in intron sequences, explaining missing band)*; (5), (11) XbaI; (6), (12) XhoI.
(13), all panels: Length marker (DNA Ladder Mix, Fermentas, Germany). (bp), base pairs.



Supplemental Figure 2.

MADS domain protein alignment of isolated AG-like genes of *C. bursa-pastoris* and of homologous members of the *AG* clade from different angiosperm species. One *AG* homolog originated from the gymnosperm *Gnetum gnemon* and served later in the phylogeny reconstruction as rooting outgroup. Complete protein sequences were aligned including N-terminal extensions.

The alignment was constructed using the MUSCLE programme (Edgar, 2004) with default parameters. The protein alignment was checked manually for correctness.

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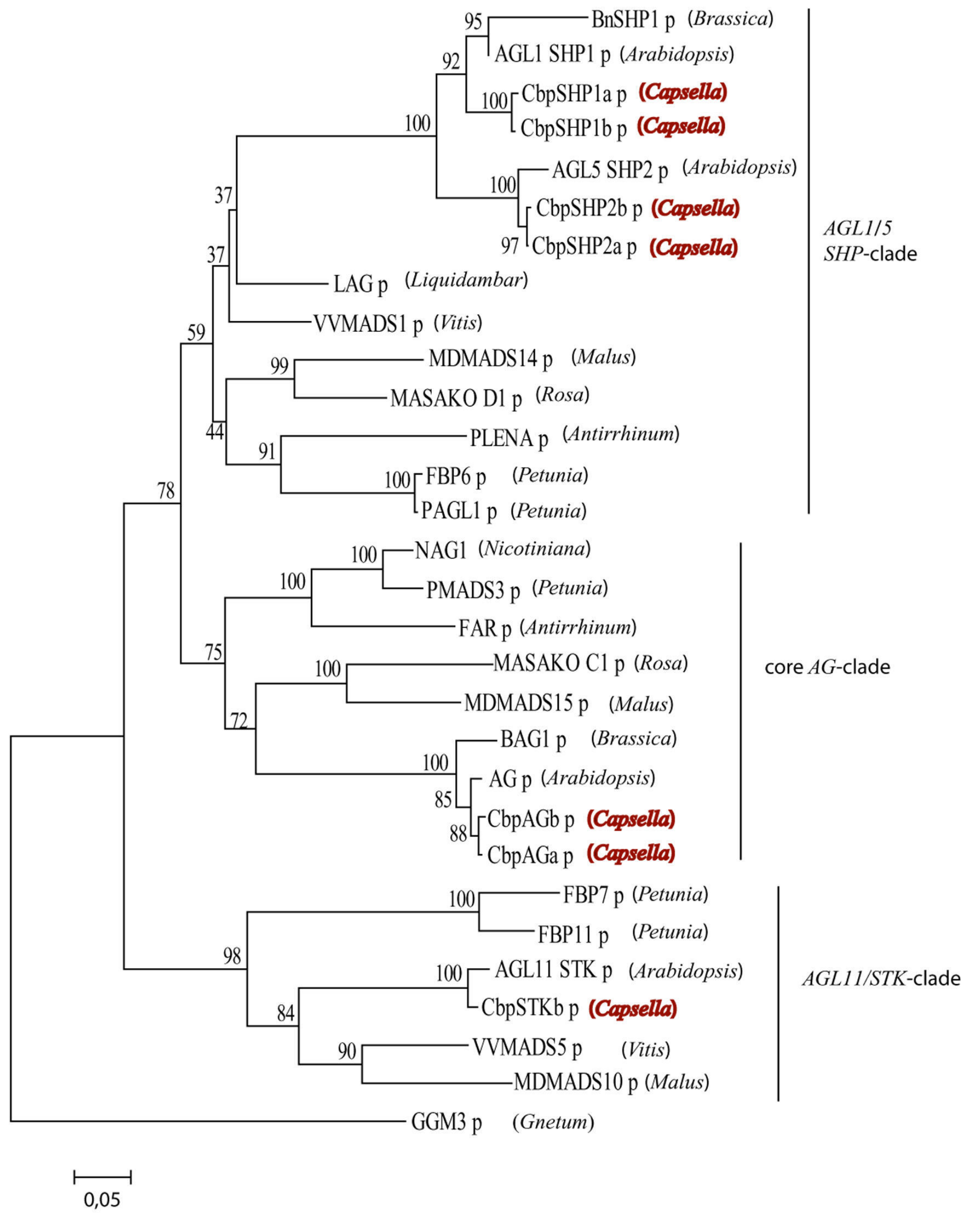

Supplemental Figure 3.

Phylogeny reconstruction of AG-like proteins of *C. bursa-pastoris* and of other angiosperm species as well as one gymnosperm species (*Gnetum gnemon*) as rooting outgroup. Phylogeny reconstruction was performed using Neighbour Joining method (Saitou and Nei, 1987) with the help of the MEGA3 software (Kumar et al., 2004). As model, poisson correction was chosen with gaps incorporated and gamma distribution of 1.0. Support values were estimated with 10000 bootstrap replicates (Felsenstein, 1985) and bootstrap-values are indicated at tree nodes. Branch lengths are equivalent to the substitution rates indicated in the scale bar; p: protein.

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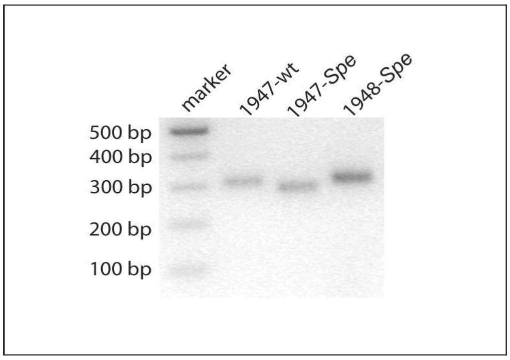
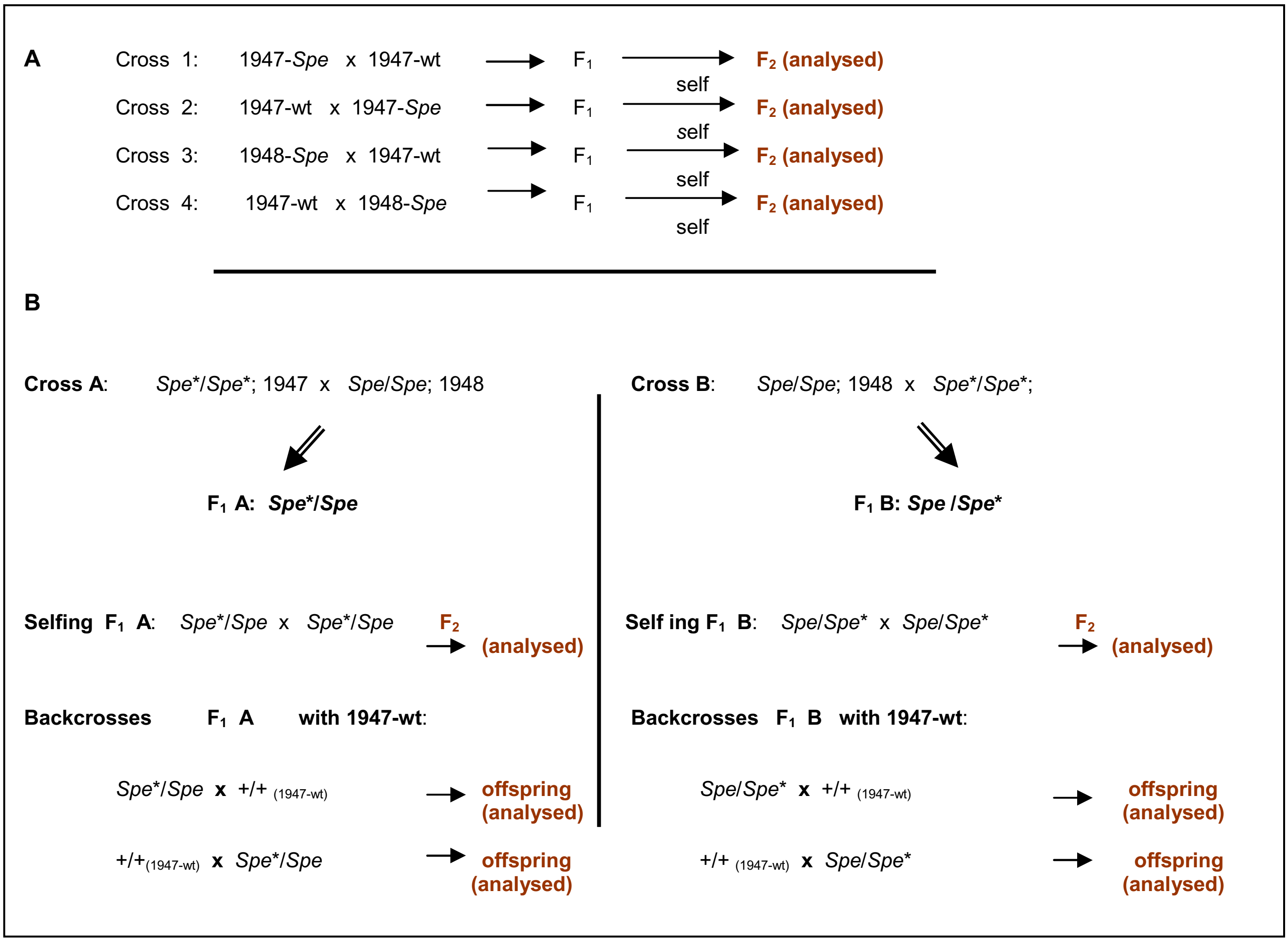


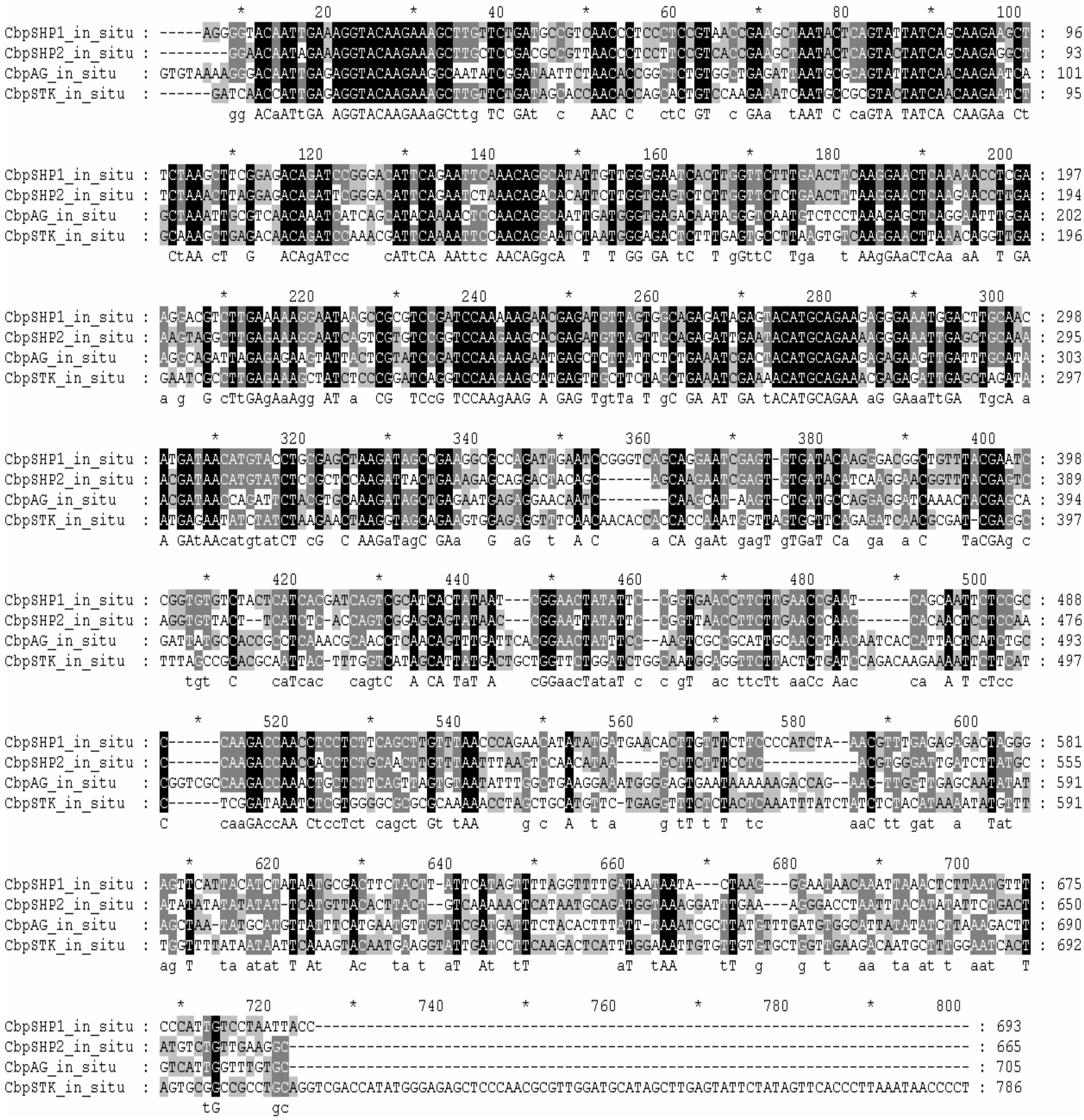
Supplemental Figure 4.

Result of a PCR experiment testing for a specific deletion in the 2nd intron of *CbpAGa* of the 1948-*Spe* line in comparison to the 1947-*Spe* line. Fragments were amplified with specific primers (Supplemental Table 4) for *CbpAGa* with a standard PCR program. Fragment lengths of 1947-wt: 299 bp, 1947-*Spe*: 277 bp, 1948-*Spe*: about 299 bp.



Supplemental Figure 5.

Crossing schedule; overview of crosses performed.
(A) Formal genetic analysis to test for the number of genetic loci responsible for the *Spe* phenotype.
(B) Analysis to test for identity (allelism) of the genetic *Spe* loci in the lines 1947-*Spe* and 1948-*Spe*, with *Spe* locus originating from line 1947 *Spe* marked with an asterisk for ancestry differentiation; +, symbol for wild-type allele.



Supplemental Figure 6.

Nucleotide alignment of *in situ* hybridization probes of *AG*-like genes. The alignment was generated with CLUSTALX (Jeanmougin et al., 1998).

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Supplemental Table 1. Test for fertility of 2nd whorl stamens.

Pollen recipient (emasculated)	Pollen donor	Plants selfed	Number of seeds/fruit
1947-wt	1947-*Spe* 2nd whorl stamens		13
1947-wt	1947-*Spe* 2nd whorl stamens		20,9
1947-wt	1948-*Spe* 2nd whorl stamens		13,2
1947-wt	1948-*Spe* 2nd whorl stamens		0,3
1947-*Spe*	1947-*Spe* 2nd whorl stamens		24,5
1947-*Spe*	1947-*Spe* 2nd whorl stamens		22,0
1948-*Spe*	1948-*Spe* 2nd whorl stamens		12,8
1948-*Spe*	1948-*Spe* 2nd whorl stamens		0,9
1947-wt	1947-*Spe* 3rd whorl stamens		17,5
1947-wt	1948-*Spe* 3rd whorl stamens		13,4
1947-*Spe*	1947-*Spe* 3rd whorl stamens		8,4
1947-wt	1947-*Spe* all stamens		19,4
1947-wt	1948-*Spe* all stamens		21,6
1947-*Spe*	1947-*Spe* all stamens		23,0
1948-*Spe*	1948-*Spe* all stamens		19,3
		1947-wt	26,6
		1947-wt	25,1
		1947-wt	22,7
		1947-*Spe*	24,8
		1948-*Spe*	22,6
		1948-*Spe*	24,0
1947-wt	Not pollinated (neg. control)		0
1947-wt	Not pollinated (neg. control)		0
1947-*Spe*	Not pollinated (neg. control)		0,2
1947-*Spe*	Not pollinated (neg. control)		0
1947-*Spe*	Not pollinated (neg. control)		2,3
1948-*Spe*	Not pollinated (neg. control)		0,4
1948-*Spe*	Not pollinated (neg. control)		0,4

Supplemental Table 2. Summary of floral development in *Capsella bursa-pastoris*: Landmark events that occur at beginning of each developmental stage are summarised and assorted in chronological order.

Developmental stage	Landmark event at begin of developmental stage.
1	Flower primordium arises at edge of inflorescence meristem as a small dome of cells.
2	Flower primordium develops by differentiation from the inflorescence meristem through a groove and through length growth in an right angle to the main apex.
3	Sepals begin to bulge out as ridges, first the axial ones followed by the lateral ones, pedicel begins to form (and will elongate concurrently to the increasing bud size in all following stages) → formation of the 1st floral organ whorl.
4	Abaxial sepal ridge lengthens and curves inward to overlie the flower primordium closely followed by the other sepals in aforementioned order.
5	Stamen primordia begin to bulge out, first the four medial stamens parallel to the axial sepals followed by the two lateral stamen primordia; the central carpel dome starts to bulge out → formation of the 3rd and 4th organ whorl.
6	Floral bud is fully enclosed by sepals, primordia of long medial stamens bulge out further and become distinct from central dome, lateral stamen primordia follow through stage duration, central dome is developing a rim growing upward to form an oval tube; petals begin to arise with small primordia in the sinuses of the sepals, → formation of 2nd floral whorl becomes visible.
7	Growing primordia of medial stamens begin to become stalked giving rise to the filament.
8	Thecae start to bulge out from adaxial anther surfaces as longitudinal ridges in medial stamens followed by the lateral ones, petal primordia still small and hemispherical.
9	Petal primordia begin to be stalked, rapid growth of stamens.
10*	The oval carpel tube is closed at the tip with a smaller circular constriction and starts to develop stigmatic papillae on top.
11*	Petal tips reach level with the tips of lateral stamens.
12	Petals reach level with the longer medial stamens, rapid length growth of all floral organs.
13	Begin of anthesis: Sepals begin to open to a small circular hole through which the already receptive stigma is growing to overtop the sepal tips. This behaviour shows clear proterogyny, like it is known from *Arabidopsis* flowers. During stage 13 sepals open completely by extrusion trough length growth of the inner organs.
14	Long anthers and petals extend the carpel tip in length; sepals petals and stamens are bracing from the carpel.
15	Sepals, petals and stamens cling to the carpel surface and by doing so long medial stamens release pollen to the carpel stigma and finally ensure pollination.
16*	Stigma extends above the long anthers, silicules bulge out at the valve tips on each side of the carpel leading to the typical triangular and later heard shaped fruit form.
17	Sepals, petals and stamens wither and start to fall off the flower.
18	Silicules turn yellow.
19	Valves separate from the dried silicules when agitated causing seed fall.

* Stages in which developmental events in flowers of *C. bursa-pastoris* deviate from the developmental stages of *Arabidopsis thaliana* flowers as described in Smyth et al. (1990).

Supplemental Table 3. Segregation patterns of F_3 generations obtained from F_2 plants with questionable intermediate phenotypes of cross 1a.

F_3 generation	wt	int/*Spe*	F_3 generation	wt	int/*Spe*	F_3 generation	wt	int/*Spe*	F_3 generation	wt	int/*Spe*
F3-4 *	0	18	F3-64	3	13	F3-112 *	0	30	F3-129 *	0	14
F3-7	3	13	F3-70	3	14	F3-115 *	0	17	F3-142 *	0	19
F3-8	6	10	F3-71	3	15	F3-116	2	15	F3-156	8	11
F3-11	6	9	F3-75	9	9	F3-121	4	5	F3-165	3	15
F3-21	5	12	F3-77	4	9	F3-122	6	9	F3-166	6	12
F3-26	1	15	F3-98	2	3	F3-126	5	12	F3-168 *	0	19
F3-41	3	3	F3-99	8	9	F3-127 *	0	19	F3-171	4	14
F3-56	4	13	F3-111	4	10	F3-128	3	14			

* F_3 generations where no segregation was observed in this analysis; they will most probably be homozygous for *Spe*, but with a lower expressivity in the *Spe* phenotype.

Supplemental Table 4. Results of phenotyping and genotyping of the parent plants 1947-wt and 1947-*Spe*, the F_1 generation and the F_2 mapping population; WT, wild-type; M, mutant (*Spe* or intermediate).

Plant	Phenotype	Genotype	Plant	Phenotype	Genotype
1947-wt	WT	C/C	F2-98	M	C/-
1947-*Spe*	M	-/-	F2-99	M	-/-
F1	M	C/-	F2-100	M	C/-
F2-1	M	C/-	F2-101	M	-/-
F2-2	M	-/-	F2-102	M	-/-
F2-3	no flower		F2-103	M	C/-
F2-4	M	C/-	F2-104	M	C/-
F2-5	M	-/-	F2-105	M	C/-
F2-6	WT	no DNA	F2-106	M	C/-
F2-7	M	-/-	F2-107	M	C/-
F2-8	M	C/-	F2-108	M	-/-
F2-9	M	C/-	F2-109	M	C/-
F2-10	M	C/-	F2-110	M	C/-
F2-11	M	C/-	F2-111	WT	C/C
F2-12	WT	C/C	F2-112	WT	C/C
F2-13	M	C/-	F2-113	WT	C/C
F2-14	M	C/-	F2-114	WT	C/C
F2-15	M	C/-	F2-115	M	-/-
F2-16	M	-/-	F2-116	M	C/-
F2-17	M	C/-	F2-117	M	C/-
F2-18	no flower		F2-118	M	C/-
F2-19	M	C/-	F2-119	M	C/-
F2-20	WT	C/C	F2-120	WT	C/C
F2-21	M	-/-	F2-121	M	C/-
F2-22	M	C/-	F2-122	WT	C/C
F2-23	M	C/-	F2-123	M	C/-
F2-24	M	C/-	F2-124	M	C/-

F2-25	M	C/-	F2-125	M	C/-
F2-26	M	C/-	F2-126	M	-/-
F2-27	WT	C/C	F2-127	M	C/-
F2-28	M	C/-	F2-128	M	-/-
F2-29	no flower		F2-129	M	-/-
F2-30	no flower		F2-130	WT	C/C
F2-31	M	C/-	F2-131	M	-/-
F2-32	M	-/-	F2-132	M	C/-
F2-33	M	C/-	F2-133	M	C/-
F2-34	WT	C/C	F2-134	M	C/-
F2-35	WT	C/C	F2-135	WT	C/C
F2-36	WT	C/C	F2-136	M	-/-
F2-37	WT	C/C	F2-137	M	C/-
F2-38	M	C/-	F2-138	M	-/-
F2-39	M	C/-	F2-139	M	-/-
F2-40	M	-/-	F2-140	M	C/-
F2-41	M	C/-	F2-141	M	-/-
F2-42	M	C/-	F2-142	WT	C/C
F2-43	M	C/-	F2-143	WT	C/C
F2-44	WT	C/C	F2-144	M	C/-
F2-45	M	C/-	F2-145	M	C/-
F2-46	M	C/-	F2-146	M	C/-
F2-47	M	C/-	F2-147	M	C/-
F2-48	M	-/-	F2-148	M	C/-
F2-49	M	C/-	F2-149	M	C/-
F2-50	WT	C/C	F2-150	WT	C/C
F2-51	M	C/-	F2-151	WT	C/C
F2-52	M	-/-	F2-152	M	C/-
F2-53	M	C/-	F2-153	WT	C/C
F2-54	M	C/-	F2-154	WT	C/C
F2-55	M	C/-	F2-155	M	-/-
F2-56	M	C/-	F2-156	M	C/-
F2-57	M	C/-	F2-157	M	C/-
F2-58	M	C/-	F2-158	WT	C/C
F2-59	M	-/-	F2-159	M	C/-
F2-60	M	C/-	F2-160	WT	C/C
F2-61	M	C/-	F2-161	WT	C/C
F2-62	M	C/-	F2-162	M	C/-
F2-63	WT	C/C	F2-163	M	-/-
F2-64	WT	C/C	F2-164	M	-/-
F2-65	M	C/-	F2-165	M	C/-
F2-66	WT	C/C	F2-166	M	C/-
F2-67	WT	C/C	F2-167	WT	C/C
F2-68	WT	C/C	F2-168	M	C/-
F2-69	M	C/-	F2-169	M	-/-
F2-70	WT	C/C	F2-170	M	-/-
F2-71	M	C/-	F2-171	M	C/-
F2-72	WT	C/C	F2-172	M	C/-
F2-73	M	C/-	F2-173	WT	C/C
F2-74	M	C/-	F2-174	WT	C/C
F2-75	M	-/-	F2-175	M	C/-
F2-76	M	C/-	F2-176	M	-/-
F2-77	WT	C/C	F2-177	M	C/-

F2-78	M	C/-	F2-178	M	C/-
F2-79	WT	C/C	F2-179	WT	C/C
F2-80	WT	C/C	F2-180	M	-/-
F2-81	M	C/-	F2-181	WT	C/C
F2-82	M	C/-	F2-182	M	-/-
F2-83	M	C/-	F2-183	M	C/-
F2-84	M	C/-	F2-184	WT	C/C
F2-85	M	C/-	F2-185	M	C/-
F2-86	WT	C/C	F2-186	M	C/-
F2-87	M	C/-	F2-187	M	-/-
F2-88	WT	C/C	F2-188	M	C/-
F2-89	M	C/-	F2-189	M	C/-
F2-90	M	-/-	F2-190	M	C/-
F2-91	M	C/-	F2-191	M	C/-
F2-92	M	C/-	F2-192	M	C/-
F2-93	M	C/-	F2-193	WT	C/C
F2-94	M	-/-	F2-194	WT	C/C
F2-95	M	C/-	F2-195	M	-/-
F2-96	WT	C/C	F2-196	M	-/-
F2-97	WT	C/C			

Supplemental Table 5. Sequences of primers used in this work.

Primers for isolation of complete mRNAs of *AGAMOUS* (*AG)*-like genes.

Primer names and fragment lengths (in parentheses)	Primer sequences (Fwd: forward and Rev: reverse direction of primer)
*CbpAG*_ges (1051, 1055 bp)	Fwd: 5´-TTA GCA CAA CCT TAC CTT CC-3´ and Rev: 5´-GCA CAA ACC AAT GAC AAG TC-3´
*CbpSHP1*_ges1 (972 bp)	Fwd: 5´-GGA ATA TAG TTT TCT CAT CAC-3´and Rev: 5´-AAG AGT TTA ATT TGT CAT TCC-3´
*CbpSHP1*_ges2 (968 bp)	Fwd: 5´-CTT TCG GTG ATG TGA TAG G-3´ and Rev: 5´-GGA ACT TTG GAT TTA AAT ATT G-3´
*CbpSHP2*_ges (1001, 1005 bp)	Fwd: 5´-TCT CTC AGA TTT CAT CTT CC-3´and Rev: 5´-GCC TTC AAC AGA CAT AGT C-3´
*CbpSTK*_ges (945 bp)	Fwd: 5´-AGA TAG AGA TAG AGW GAG AG3´- and Rev: 5´-CCA AAG CAT TGT CTT CAA CC-3´

Primers for generation of genomic template fragments for Southern hybridization, fragments spanning 4-5 introns from I-domain to K-domain of *AG*-clade genes.

*CbpAG*_IKC (966-984 bp)	Fwd: 5´-GGA GAA TTC TAA CAC CGG CTC TGT G-3´ and Rev: 5´AAC TCT AGA GCA GTT TGG TCT TGG CG-3´
*CbpSHP1*_IKC (1188 bp)	Fwd: 5´-GTTCTC GAG CCG TCA ACC CTC CCT CC-3´ and Rev: 5´-GAT TCT AGA GAT GCG ACT GAT CGT GAT G -3´
*CbpSHP2*_IKC (877-879 bp)	Fwd: 5´-AAG AAT TCT TGC TCC GAC GCC GTT AAC-3´ and Rev: 5´-GGT CTA GAT TGG AGG AGT TGT GGT TGG-3´
*CbpSTK*_IKC (877-906 bp)	Fwd: 5´-CAA CTC GAG CAC TGT CCA AGA AAT CAA TGC-3´ and Rev: 5´-TGT CTA GAT CAG AGT AAG AAC CTC CAT TGC-3´

Primers for isolation non-*AG*-clade genes for *in situ* hybridization.

*CbpH4*_3´end (RT) (535~ bp)	5´-CTC TTC TAG AGG ATT GGG AAA GGG AGG A-3´
*CbpAP3*_3RACE (744 bp + poly-A)	5´-CAC CAC AAC GAA GGA GAT C-3´
*CbpPI*_3RACE (820, 830 bp + poly-A)	5´-G GAA GGT GGG AGT AGT CAC-3´
*CbpAP1*_3RACE (789, 792 bp + poly-A)	5´-CTT ATT GCA CCT GAG TCC G-3´

Primers for construction of template fragments for in vitro transcription of *in situ* hybridization probes with T7-RNA-polymerase-promoter in the forward- and restriction site (XbaI, XhoI) in the reverse-primer.

CbpAG antisense (705 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG CAC AAA CCA ATG ACA AGT C-3´ and Rev: 5´-AGTA TCT AGA GTG TAA AAG GGA CAA TTG AGAG-3´
CbpAG sense (705 bp	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG TGT AAA AGG GAC AAT TGA GAG-3´ and Rev: 5´-ACTA TCT AGA GCA CAA ACC AAT GAC AAG TC-3´
CbpSHP1 antisense (713 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGA ACT TTG GAT TTA AAT ATT G-3´ and Rev: 5´-AGTC TCT AGA AGG GGT ACA ATT GAA AGG TAC-3´
CbpSHP1 sense (713 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG TAC AAT TGA AAG GTA C-3´ and Rev: 5´-AGTC TCT AGA GGA ACT TTG GAT TTA AAT ATT G-3´
CbpSHP2 antisense (691 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGG CCT TCA ACA GAC ATA GTC-3´ and Rev: 5´-AGTA TCT AGA GGA ACA ATA GAA AGG TAC AAG-3´
CbpSHP2 sense (691 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGA ACA ATA GAA AGG TAC AAG-3´ and Rev: 5´-ATCT TCT AGA GCC TTC AAC AGA CAT AGT C-3´
CbpSTK antisense (715 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGC CAA AGC ATT GTC TTC AAC C-3´ and Rev: 5´-AGCT TCT AGA GAT CAA CCA TTG AGA GGT AC-3´
CbpSTK sense (714bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGA TCA ACC ATT GAG AGG TAC-3´ and Rev: 5´-ACTA TCT AGA CCA AAG CAT TGT CTT CAA CC-3´
CbpAP1 antisense (820 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGT TAT TGC ACC TGA GTC CGA C-3´ and Rev: 5´-TAGC CTC GAG TTC GTT CTC TCC AAC CTT C-3´
CbpAP1 sense (820 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGT TCG TTC TCT CCA ACC TTC-3´ and Rev: 5´- TAGC CTC GAG TTA TTG CAC CTG AGT CCG AC-3´
CbpAP3 antisense (694 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GG GAA GTC TTG AAT ACA TTC CAC-3´ and Rev: 5´-TTCA TCT AGA CTG ATG TCG ATG TTT GGA G-3´
CbpAP3 sense (694 bp)	Fwd: 5´-TAA TAC GAC TCA CTA TAG GGC TGA TGT CGA TGT TTG GAG-3´ and Rev: 5´-TTCA TCT AGA GAA GTC TTG AAT ACA TTC CAC-3´
CbpPI antisense (616 bp)	Fwd: 5´- TAA TAC GAC TCA CTA TAG GGA AGC AAA CAC ACC ACA TGC-3´ and Rev: 5´-TAGC CTC GAG ATG CTA AGC ATG AGA ACC-3
CbpPI sense (616 bp)	Fwd: ´5´- TAA TAC GAC TCA CTA TAG GGA TGC TAA GCA TGA GAA CC-3´and Rev: 5´-TAGC CTC GAG AAG CAA ACA CAC CAC ATG C

Primers for amplification of fragments from genomic DNA used for genotyping with pyrosequencing. Marked in red: primer biotinylated at 5´end.

CbpAGa	Fwd: 5’ CTA TGT TCT TCT TTT TCG GTT TCC T 3’ and
(486 bp, 1st intron)	Rev: 5’ TA GGG CTA AAC TGA TTA AAC ATC 3’
CbpAGb	Fwd: 5’ TGA TCA TAC AAC ACT AGA CAT GTG 3’ and
(751 bp, 2nd intron)	Rev: 5’ TAA ACT TTA CTT TTC TGT TTC GTT GC 3’
CbpSHP1a	Fwd: 5’ TGT ATA CAA ATG GTG GCA TTC TGA AG 3’ and
(544 bp, 4th intron)	Rev: 5’ AG ATC GAG AGA GAG AGG TAC ACA C 3’
CbpSHP1b	Fwd: 5’ CGA TTA GAC TCG GTT TTG GCA TGG 3’ and
(297 bp, promoter)	Rev: 5’ AGG ACA TTG AAG GAG GAC CAT CC 3’
CbpSHP2a	Fwd: 5’ GCT AGT GAT CAT TTT TTT CTT GTT GAA G 3’ and
(535 bp, 2nd intron)	Rev: 5’ TGT CTC GGA CTA AAT ACC AAC G 3’
CbpSHP2b	Fwd: 5’ GC ATG TGA CAT GAC TTA ATA GTA CC 3’ and
(482 bp, 2nd intron)	Rev: 5’ GGT TCT AAA ACA CTA CTA ACT GGA C 3’
CbpSTKa	Fwd: 5’ GAT AGG GTT CGT TCA TCA TCC AC 3’ and
(419 bp, promoter)	Rev: 5’ ATA ACT CCT TGC TTA CAT GCG AC 3’
CbpSTKb	Fwd: 5’ CAC TTG TTT GAT CGT CTC ATC ATC AAG 3’ and
(583 bp, 1st intron)	Rev: 5’ AAT AAG GGG AGA GAG AGA GAG AAA C 3’

Primers used in the pyrosequencing method.

CbpAGa	5’ TAA AAC AAG TTA AAC TAA AAC CAA ATC TTT 3’
CbpAGb	5’ ACA AGT ACG TTC CAT TAT TTT CTA TC 3’
CbpSHP1a	5’ AAA TAA TCA AGT TAA GGT ACA AAG ATA G 3’
CbpSHP1b	5’ TAG ACT CCT TGT AGT CCT AGC 3’
CbpSHP2a	5’ TAT GAT CTT ATT AGT CAA GTC TCA TAT 3’
CbpSHP2b	5’ GAA GTG AAA TTT GTA AAT ACT TTG CCA 3’
CbpSTKa	5’ AAT AAA CTC TTG TTA GTC ACT ATC GA 3’
CbpSTKb	5’ TCT GTG TAA TAA TGT TTC TTG TGT TG 3’

Primers used in the test for a deletion in the different *SPE* lines.

*CbpAGa*_del_fwd (277-299bp)	5´AGT TTT GAT CAT ACA ACA CTA CAC ATG TC 3´
*CbpAGa*_del_rev (277-299bp)	5´GAA ATC TCA AAT CTT TTA TGG TTG GAG ATG 3´

Supplemental Table 6. GenBank/EMBL accession numbers of isolated genes.

Gene name	Sequence character	GenBank/EMBL Accession number
CbpH4	Partial cds, part of 3´UTR	EU551759
CbpAP1a	Partial cds & 3´UTR	EU551760
CbpAP1b	Partial cds & 3´UTR	EU551761
CbpPIa	Partial cds & 3´UTR	EU551762
CbpPIb	Partial cds & 3´UTR	EU551763
CbpAP3a	Partial cds & 3´UTR	EU551764
CbpAP3b	Partial cds & 3´UTR	EU551765
CbpSTKa	5´UTR, partial cds	EU551766
CbpSTKb,	5´UTR, complete cds, 3´UTR	EU551767
CbpSHP2a	5´UTR, complete cds, 3´UTR	EU551768
CbpSHP2b	5´UTR, complete cds, 3´UTR	EU551769
CbpSHP1a,	5´UTR, complete cds, 3´UTR	EU551770
CbpSHP1b,	5´UTR, complete cds, 3´UTR	EU551771
CbpAGa,	5´UTR, complete cds, 3´UTR	EU551772
CbpAGb,	5´UTR, complete cds, 3´UTR	EU551773
CbpAGa_Spe	Partial genomic DNA	EU662251
CbpAGa_wt	Partial genomic DNA	EU662252
CbpAGb_Spe	Partial genomic DNA	EU662253
CbpAGb_wt	Partial genomic DNA	EU662254
CbpSHP1a_Spe	Partial genomic DNA	EU662255
CbpSHP1a_wt	Partial genomic DNA	EU662256
CbpSHP1b_Spe	Partial genomic DNA	EU662257
CbpSHP1b_wt	Partial genomic DNA	EU662258
CbpSHP2a_Spe	Partial genomic DNA	EU662259
CbpSHP2a_wt	Partial genomic DNA	EU662260
CbpSHP2b_Spe	Partial genomic DNA	EU662261
CbpSHP2b_wt	Partial genomic DNA	EU662262
CbpSTKa_Spe	Partial genomic DNA	EU662263
CbpSTKa_wt	Partial genomic DNA	EU662264
CbpSTKb_Spe	Partial genomic DNA	EU662265
CbpSTKb_wt	Partial genomic DNA	EU662266

Lebenslauf

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07/1983 - 06/1992	Hüffertgymnasium, Warburg Abschluss: Abitur (Note: 2,2)

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09/1992 - 09/1995	Ausbildung zur Theatermalerin an den Städtischen Bühnen Münster mit Abschlußprüfung durch die Paritätische Prüfungskommission in Berlin

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10/1995 - 08/1997	Grundstudium Biologie und Deutsch, (Lehramt, Sek.II), Westfälische Wilhelms-Universität Münster
10/1997 - 08/2001	Hauptstudium Biologie (Diplom), Westfälische Wilhelms-Universität Münster
05/2000 - 06/2001	Diplomarbeit am Institut für Botanik und Botanischer Garten an der Westfälischen Wilhelms-Universität Münster mit dem Titel : „Checkliste der Gattung *Hoya* auf Borneo (Apocynaceae – Asclepiadaceae) “ betreut durch Prof. Dr. Focke Albers
8/2001	Diplom mit der Note „sehr gut“ (1,05)

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1999 – 2001	Studentische Hilfskraft am Institut für Botanik, WWU Münster (Betreuung der botanischen Grundpraktika)
02-03/2002	Wissenschaftliche Hilfskraft am Lehrstuhl für Genetik, FSU Jena
04-06/2002	Wissenschaftliche Mitarbeiterin am Lehrstuhl für Genetik, FSU Jena; Trainings- und Forschungsaufenthalt am Max-Planck-Institut für Züchtungsforschung in Köln
07/2002 – 10/2008	Wissenschaftliche Mitarbeiterin am Lehrstuhl für Genetik, FSU Jena mit dem Promotionsthema: „Untersuchungen an einer bestandsbildenden homöotischen Blütenmutante von *Capsella bursa-pastoris* (L) Medik.“

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02/1997 – 04/1997	Ökologisches Praktikum auf der Biologischen Station Bilsa der Organisation Fundación Jatun Sacha, Ecuador
06/2000 – 06/2001	Forschungsaufenthalte an den Herbarien von Leiden (Niederlande), Kew (Großbritannien), Hamburg and Berlin im Rahmen der Diplomarbeit
09/2001 – 10/2001	Forschungsreise nach Borneo (Malaysia) mit Aufenthalten an Herbarien in Malaysia und Singapur

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Dipl. Biol. Pia Nutt

Wissenschaftliche Publikationen

P. Nutt, J. Ziermann, M. Hintz et al. (2006): *Capsella* as a model system to study the evolutionary relevance of floral homeotic mutants, Pl. Syst. Evol. 259, pp 217-235 (Übersichtsartikel)

M. Hintz, C. Bartholmes, P. Nutt et al. (2006): Catching a ´hopeful monster´: Shepherd´s purse (*Capsella bursa-pastoris*) as a model system to study the evolution of floral development, J. Experim. Bot. 57 (13), pp 3531-3542 (Übersichtsartikel)

Conny Bartholmes, Pia Nutt, and Günter Theißen (2008):
Germline transformation of Shepherd's purse (Capsella bursa-pastoris) by the 'floral dip' method as a tool for evolutionary and developmental biology, Gene 409, pp 11-19 (Originalarbeit)

„Pia Nutt[1], Janine Ziermann[1] and Günter Theißen (submitted):
Ectopic expression and co-segregation of an *AGAMOUS* orthologue in *Stamenoid petals*, a natural floral homeotic variant of *Capsella bursa-pastoris.*
([1]These authors contributed equally to this work)" (Originalarbeit)

Wissenschaftliche Vorträge

Analysis of a naturally occurring floral homeotic mutant of the shepherd´s purse *(Capsella bursa-pastoris)*;
2nd German Middle East Plant Molecular Biology Meeting, Jena, Germany (2004)

Die molekularen Grundlagen der Blütenentwicklung, oder *wie die Blütenpflanzen ihr ABC lernen;*
Treffen der AG Zierpflanzen der Gesellschaft für Pflanzenzüchtung (GPZ), Hillscheid, Germany (2006)

Investigating the evolutionary potential of a floral homeotic variety of *Capsella;*
First and Founding Meeting of the European Society for Evolutionary Developmental Biology, Prague, Cech Republic (2006)

The ABC beyond *Arabidopsis*: Ectopic expression of an orthologue of *AGAMOUS* in a natural floral homeotic variety of *Capsella bursa-pastoris* (shepherd's purse);
Mitteldeutsches Pflanzenphysiologie-Treffen and "Symposium on Plant signal transduction" (SFB 604), Jena, Germany (2008)

Wissenschaftliche Kongressbeiträge in Form von Postern

P. Nutt, K. Hehmann, T. Steinke, G. Schüttler, G. Theißen, B. Neuffer: Decandric forms in *Capsella bursa-pastoris* (L.) Medik. (Brassicaceae) - characterisation and candidate genes; 16th International Symposium, Biodiversity and Evolutionary Biology Frankfurt/Main, Germany (2003)

P. Nutt, B. Neuffer & Guenter Theißen: A floral homeotic variety of *Capsella* in the wild; Summer Research Conference, Mechanisms in Plant Development, Saxtons River, USA (2004). (fundet by a LUBOM grand, University of Jena)

P. Nutt, B. Neuffer, G. Theißen: A floral homeotic variety of Capsella: evolutionary aspects; XVIIth International Botanical Conference, Vienna, Austria (2005).

P. Nutt, B. Neuffer, G. Theißen: A floral homeotic variety of Capsella: evolutionary aspects; NSF-DFG Conference, Understanding Species Diversity on Earth, Washington DC, USA (2005)

S.Hameister, P. Nutt, J. Ziemann, G. Theißen, B. Neuffer: 6+4=10: Decandric forms of *Capsella bursa-pastoris* (L.) Medik., characterising a floral variety of shepherd's purse; 17th International Symposium "Biodiversity and Evolutionary Biology, Bonn, Germany (2006)

Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist und ich die vorliegende Arbeit selbst angefertigt habe. Alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen habe ich in meiner Arbeit angegeben. Bei der Auswertung des Materials sowie bei der Herstellung des Manuskriptes haben mich die in der Danksagung auf Seite 112 der Dissertation genannten Personen unterstützt. Ferner erkläre ich ehrenwörtlich, für die Anfertigung der Arbeit keinen Promotionsberater in Anspruch genommen zu haben, und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Dissertation habe ich bisher nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung vorgelegt. Auch habe ich weder diese Dissertation noch eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht.

Ort, Datum Pia Nutt